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I hereby certify that this Transmittal letter, enclosed application, and any other documents referred to as enclosed herein, are being deposited in an envelope with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

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Attorney Docket No. TSRI 651.3

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Sir:

Transmitted herewith for filing is the utility patent application of inventor(s): **Cheresh, David A.; Paul, Robert; Eliceiri, Brian**

and entitled: **METHOD USEFUL FOR TREATING VASCULAR LEAKAGE AND EDEMA USING SRC or YES TYROSINE KINASE INHIBITORS**

1. **Type Of Application**

This application is:

__ an original (nonprovisional) application.
__ a divisional of prior application Serial No. _____.
__ a continuation of prior application Serial No. _____.
X a continuation-in-part of prior application Serial No. 09/470,881 filed December 22, 1999 which claims priority to Intl. Patent Application No. PCT/US99/11780 filed May 28, 1999 which claims priority to U.S. Provisional Application for Patent Serial No. 60/087,220 filed May 29, 1998.

__ The entire disclosure of the prior application is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

__ The prior application is assigned of record to:_____

__ Additional prior application information: Examiner _____ Group _____

2. Enclosed **Application Elements** are:

X A duplicate copy of this transmittal letter,
X specification (including claims and abstract) containing pages 1-96,
X drawings: __ 1 copy of _____ sheet(s) of formal drawings, **OR**
X 1 copy of Fourteen (14) sheet(s) of informal drawings (Figs. 1-13).
__ an executed declaration or oath for the utility patent application including a power of attorney, **OR**
X an unexecuted declaration or oath for the utility patent application including a power of attorney, **OR**
__ a copy of an executed declaration or oath including power of attorney from a priority application,
__ statement deleting inventor(s) named in the priority application

3. Enclosed **Accompanying Application Parts** are:

__ One(1) Verified Statement(s) relating to small entity status.
__ Copy of verified statement filed in prior application; status still proper and desired.
__ Preliminary Amendment
__ Claim cancellations are indicated in Preliminary Amendment
__ one itemized, stamped, and self-addressed postcard for the PTO Mail Room date stamp.
__ English translation document
__ Information Disclosure Statement including Form PTO-1449 and copies of the citations therein.

4. Filing Fees (as calculated below)

	(Col. 1)	(Col. 2)		
For:	Number Filed	Number Extra	Rate	Fee
Basic Fee				$ 690
Total Claims	31 — 20	= 11	x $ 18 =	$ 198
Independent Claims	3 — 3	= 0	x $ 78 =	$ 0
Multiple Dependent Claim Presented (if applicable)			+ $260 =	$ 0
			Subtotal	$ 888
			Reduction by 50% for filing by small entity	
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__ IF CHECKED, please charge our Deposit Account No. _____ in the amount of $_____.
X A check in the amount of $ 888.00 to cover the filing fee is enclosed.
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X The Commissioner is authorized to charge payment of the following amounts associated with this communication or credit any overpayment to Deposit Account No. _____;
X Additional filing fees under 37 CFR 1.16 or deficiencies in remittances therefor.
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[Begin Extension Option]
_ The Commissioner is hereby generally authorized under 37 CFR 1.136(a)(3) to treat any future reply in this or any related application filed pursuant to 37 CFR 1.53 requiring an extension of time as incorporating a request therefor, and the Commissioner is hereby specifically authorized to charge Deposit Account No. _____ for any fee that may be due in connection with such a request for an extension of time.
[End Extension Option]

Date: March 29, 2000

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METHODS USEFUL FOR TREATING VASCULAR LEAKAGE AND EDEMA USING SRC or YES TYROSINE KINASE INHIBITORS

Cross-reference to Related Applications

This application claims priority to U.S. Patent Application Serial No. 09/470,881, filed Dec. 22, 1999, which claims priority to International Patent Application Number PCT/US99/11780, designating the United States of America and filed May 28, 1999, which claims priority to United States Provisional Application for Patent Serial No. 60/087,220 filed May 29, 1998.

Statement of Government Rights

Some of the work disclosed has been supported in part by grants from the NIH on behalf of The United States of America. Therefore, the government of the United States of America may have certain rights in the invention.

Technical Field

The present invention relates generally to the field of medicine, and relates specifically to methods useful in treating disease pathology associated with blood vessel leakage and/or edema by modulating vascular permeability.

Background

In response to trauma, disease, or inflammation, blood vessels are subject to signals which induce an increase in vascular permeability and which can lead to edema, inflammation and other pathological complications. In response to cancer cell stimulation during tumor formation, blood vessels can be induced to become permeable and undergo angiogenesis, the formation of new blood vessels to

vascularize the growing tumor cells, however, the resultant pathological tissue damage is due to tumor formation, and not vascular leakage and/or edema.

It has been previously reported that angiogenesis depends on the interaction between vascular integrins and extracellular matrix proteins. Brooks et al., Science, 264:569-571 (1994). It was reported that programmed cell death (apoptosis) of angiogenic vascular cells is initiated by the interaction, which would be inhibited by certain antagonists of the vascular integrin $\alpha_v\beta_3$. Brooks et al., Cell, 79:1157-1164 (1994). More recently, it has been reported that the binding of matrix metalloproteinase-2 (MMP-2) to vitronectin receptor ($\alpha_v\beta_5$) can be inhibited using $\alpha_v\beta_5$ antagonists, and thereby inhibit the enzymatic function of the proteinase. Brooks et al., Cell, 85:683-693 (1996). The α_v integrins have been identified as important components in endothelial cell survival in angiogenic blood vessels. Specific integrin α_v integrin antagonists block discrete growth-factor induced angiogenesis pathways. For example, vascular endothelial growth factor (VEGF)-induced angiogenesis is blocked by integrin $\alpha_v\beta_5$ antagonists, while basic fibroblast growth factor (bFGF)-induced angiogenesis is blocked by integrin $\alpha_v\beta_3$ antagonists.

A requirement for Src tyrosine kinase activity for VEGF- but not bFGF- induced angiogenesis demonstrated that significant differences in regulation and activation signals between these pathways exist, in both chick embryo and mouse models. Eliceiri et al., Molecular Cell, 4: 915-924 (1999).

Changes in vascular permeability due to angiogenic signals from tumor cells have provided a model for examining

the signal pathways related to cancer, however, vascular permeability due to injury, disease or other trauma to the blood vessels is a major cause of vascular leakage and edema associated with tissue damage. For example, cerebrovascular disease associated with cerebrovascular accident (CVA) or other vascular injury in the brain or spinal tissues are the most common cause of neurologic disorder, and a major source of disability. Typically, damage to the brain or spinal tissue in the region of a CVA involves vascular leakage and/or edema. Typically, CVA can include injury caused by brain ischemia, interruption of normal blood flow to the brain; cerebral insufficiency due to transient disturbances in blood flow; infarction, due to embolism or thrombosis of the intra- or extracranial arteries; hemorrhage; and arteriovenous malformations. Ischemic stroke and cerebral hemorrhage can develop abruptly, and the impact of the incident generally reflects the area of the brain damaged. (See The Merck Manual, 16th ed. Chp. 123, 1992).

Other than CVA, central nervous system (CNS) infections or disease can also effect the blood vessels of the brain and spinal column, and can involve inflammation and edema, as in for example bacterial meningitis, viral encephalitis, and brain abscess formation. (See The Merck Manual, 16th ed. Chp. 125, 1992). Systemic disease conditions can also weaken blood vessels and lead to vessel leakage and edema, such as diabetes, kidney disease, atherosclerosis, and the like. Thus, vascular leakage and edema are critical pathologies, distinct from and independent of cancer, which are in need of effective specific therapeutic intervention in association with a variety of injury, trauma or disease conditions.

We have discovered that selective inhibition of Src family tyrosine kinase activity reduces injury or trauma associated VP increase in tissues, and results in amelioration of pathology related to blood vessel leakage and/or edema.

Summary of the Invention

Tissue damage related to vascular leakage and/or edema associated with deleterious changes in vascular permeability can be ameliorated by a Src family tyrosine kinase inhibitor. To that end, an effective, vascular permeability modulating amount of a Src family tyrosine kinase inhibitor is administered to a tissue in need of such treatment. Tissue damage due to vascular leakage or edema can be reduced in this manner.

In particular, the present invention provides a method for inhibiting vascular permeability increase in a tissue suffering from a disease condition which is associated with vascular leakage and/or edema by contacting said tissue with a therapeutically effective, vascular permeability inhibiting amount of a Src family tyrosine kinase inhibitor together with a pharmaceutically acceptable carrier therefor. In a preferred embodiment, a Src specific tyrosine kinase inhibitor is administered to the tissue.

Any pathology which involves deleterious injury-induced increase in vascular permeability and tissue damage due to vascular leakage or edema can be treated by this method. Such pathological events can include trauma to the blood vessels such as physical ligation, blockage, separation, occlusion, trauma, and the like. Other systemic pathological events such as atherosclerosis, diabetic retinopathy, inflammatory disease

due to infection by microbial agents, arthritis and the like are also appropriately treated by a method of the invention.

The methods of the present invention are useful for treating cerebrovascular disease or trauma by ameliorating tissue damage due to increased vascular leakage and/or edema associated therewith. In particular, the methods of the present invention are useful for ameliorating tissue damage associated with Vascular Endothelial Growth Factor (VEGF)-induced Src mediated increase in vascular permeability. However, the methods of the invention are not limited to VEGF-induced increases in vascular permeability, and are also appropriate for modulating Src family tyrosine kinase mediated increase in vascular permeability in response to other regulatory signals.

In particular, by inhibiting tyrosine kinase Src, (also referred to generically herein as Src), and the closely related tyrosine kinase Yes, (also referred to generically herein as Yes) treated tissues can be specifically modulated to inhibit therein an increase in vascular permeability associated with injury or disease.

A suitable Src family tyrosine kinase inhibitor for purposes of the present invention is a chemical inhibitor selected from the group consisting of PP1, PP2, PD173955, AGL1872, PD162531, Radicicol R2146, and Geldanamycin. Other chemical inhibitors of Src family tyrosine kinases are also appropriate for use in the methods of the invention.

Vascular permeability in tissue can also be modulated by administering to the tissue a Src family tyrosine kinase inhibitor that is a protein inhibitor, such as an inactive Src protein like Src K295M or Src 251, or an inactive Yes protein,

or an active c-terminal Src Kinase (CSK) protein.

Also suitable for vascular permeability modulation in a tissue is a nucleic acid encoding for a Src family tyrosine kinase inhibitor protein, such as an inactive Src, inactive yes or active CSK protein. Such nucleic acid inhibitors of Src family tyrosine kinase activity can encompass one or more retroviral expression vector, non-viral expression vector or the like. Such nucleic acid inhibitors may comprise the appropriate regulatory signals, such as promoters or enhancers for one or more expressible segment of nucleic acid on any given nucleic acid.

In a further aspect of the present invention, articles of manufacture comprise packaging material and a pharmaceutical composition contained within said packaging material, wherein said pharmaceutical composition is capable of modulating vascular permeability in a tissue suffering from a disease condition. The packaging material comprises a label which indicates that said pharmaceutical composition can be used for treating vascular leakage or edema associated disease conditions and the pharmaceutical composition comprises a therapeutically effective amount of Src family tyrosine kinase inhibitor in a pharmaceutically acceptable carrier.

An article of manufacture of the invention may contain as part of the pharmaceutical composition a Src family tyrosine kinase inhibitor that is a chemical inhibitor. In particular, a preferred chemical Src family tyrosine kinase inhibitor is selected from the group consisting of PP1, PP2, PD173955, AGL1872, PD162531, Radicicol R2146, and Geldanamycin, or compounds with similar Src inhibiting activity. A most preferred inhibitor is PP1.

An article of manufacture of the invention also encompasses where said pharmaceutical composition comprises a protein Src family tyrosine kinase inhibitor which is an inactive Src protein such as Src K295M or Src 251, inactive yes protein, or active CSK protein.

Alternatively, the pharmaceutical composition comprises a nucleic acid encoding for a Src family tyrosine kinase inhibitor, in a pharmaceutically acceptable carrier. In such a pharmaceutical composition, the inhibitor for which said nucleic acid encodes can be inactive Src protein, such as Src K295M or Src 251, inactive Yes protein, or active CSK protein.

Articles of manufacture may include one or more pharmaceutical compositions that contain therapeutic Src family tyrosine kinase inhibitors, or sub-therapeutic amounts of more than one Src family tyrosine kinase inhibitors, in a pharmaceutically acceptable carrier.

Pharmaceutical compositions of the articles of manufacture of the invention may comprise mixtures of one or more sub-therapeutically effective VP modulating amount of a Src family tyrosine kinase inhibitor, which act together to provide a VP reducing effect on treated tissue. The pharmaceutical composition of the article of manufacture can vary depending upon the desired modulatory effect, and the packaging labeling will correspondingly vary as well.

Brief Description of the Drawings

In the drawings forming a portion of this disclosure:

Figure 1 is a cDNA sequence of chicken c-SRC which is the complete coding sequence with the introns deleted as first described by Takeya et al., Cell, 32:881-890 (1983). The

sequence is accessible through GenBank Accession Number J00844. The sequence contains 1759 nucleotides with the protein coding portion beginning and ending at the respective nucleotide positions 112 and 1713 (SEQ ID NO. 2).

Figure 2 is the encoded amino acid residue sequence of chicken c-SRC of the coding sequence shown in Figure 1 (SEQ ID NO. 3).

Figure 3 is a cDNA sequence of human c-SRC which as first described by Braeuninger et al., Proc. Natl. Acad. Sci., USA, 88:10411-10415 (1991). The sequence is accessible through GenBank Accession Number X59932 X71157. The sequence contains 2187 nucleotides with the protein coding portion beginning and ending at the respective nucleotide positions 134 and 1486 (SEQ ID NO. 4).

Figure 4 is the encoded amino acid residue sequence of human c-SRC of the coding sequence shown in Figure 3 (SEQ ID NO. 5).

Figure 5 illustrates the activation of endogenous Src by bFGF or VEGF as described below. The top portion of the figure indicates the results of an in vitro kinase assay with the fold activation of endogenous c-SRC by either bFGF and VEGF. The bottom of the figure is the kinase assay blot probed with an anti-Src antibody as a loading control for equivalent Src and IgG content.

Figure 6 illustrates the retroviral expression of c-SRC A in activating vascular MAP kinase phosphorylation. Figure 6A shows tissue extracts of 10 day-old chick CAMs that had been exposed to VEGF or PMA for 30 minutes or infected with c-SRC A retrovirus for 48 hours. NT stands for no treatment. Src was immunoprecipitated from equivalent amounts of total protein

extract and subjected to an in vitro immune complex kinase assay using a FAK-GST fusion protein as a substrate, electrophoresed and transferred to nitrocellulose. Aliquots of the above whole tissue lysates were also measured for endogenous ERK phosphorylation by immunoblotting with an anti-phospho-ERK antibody. Figure 6B shows 10 day old CAMs that were infected with either mock RCAS or RCAS containing SRC A. After two days, CAMs were dissected, cryopreserved in OCT and sectioned at 4 μm. Sections were immunostained with an anti-phosphorylated ERK antibody (New England Biolabs), washed and detected with a goat anti-rabbit FITC-conjugated secondary antibody. Florescent images were captured on a cooled-CCD camera (Princeton Inst.)

Figure 7 is a diagram illustrating a restriction map of the RCASBP (RCAS) vector construct.

Figure 8 depicts the encoded amino acid residue sequence of human c-Yes protein in single letter amino acid representation (SEQ ID NO. 8).

Figure 9 depicts the nucleic acid sequence of a cDNA encoding for human c-Yes protein. The sequence is accessible through GenBank Accession Number M15990. The sequence contains 4517 nucleotides with the protein coding portion beginning and ending at the respective nucleotide positions 208 and 1839, and translating into to amino acid depicted in Figure 8 (SEQ ID NO. 7).

Figure 10 depicts results from retroviral delivery of Src 251 and CSK in a subcutaneous murine angiogenesis model. Figure 10A illustrates immunoblotting results for detecting flk expression. Figure 10B illustrates immunoblotting results from assay for flk under VEGF and bFGF stimulated conditions.

Figure 10C is a graph which plots the number of CD34 positive blood vessels (average of triplicate random fields at 20x) by treatment as stimulated by VEGF and bFGF in the presence of GFP, Src 251, or CSK retrovirus.

Figure 11 illustrates results from a modified Miles assay for VP of VEGF in the skin of mice deficient in src, fyn and yes. Figure 11A are photographs of treated ears. Figure 11B are graphs of experimental results for stimulation of the various deficient mice. Figure 11C plots the amount of eluted Evan's blue dye by treatment.

Figure 12 is a graph depicting the relative size of infarct in Src +/-, Src -/-, wild type (WT), and PP1 treated wild type mice. PP1 treatment consisted of 1.5 mg/kg body weight.

Figure 13 depicts sequential MRI scans of control and PP1 treated mouse brains showing less brain infarction in PP1 treated animal (right) than in the control animal (left).

Detailed Description of the Invention

A. Definitions

Amino Acid Residue: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard

polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 CFR §1.822(b)(2)).

It should be noted that all amino acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues.

Polypeptide: refers to a linear series of amino acid residues connected to one another by peptide bonds between the alpha-amino group and carboxy group of contiguous amino acid residues.

Peptide: as used herein refers to a linear series of no more than about 50 amino acid residues connected one to the other as in a polypeptide.

Cyclic peptide: refers to a compound having a heteroatom ring structure that includes several amide bonds as in a typical peptide. The cyclic peptide can be a homodetic "head to tail" cyclized linear polypeptide in which a linear peptide's n-terminus has formed an amide bond with the c-terminal carboxylate of the linear peptide, or it can contain a ring structure in which the polymer is heterodetic and comprises amide bonds and/or other bonds to close the ring, such as disulfide bridges, thioesters, thioamides, guanidino, and the like linkages.

Protein: refers to a linear series of greater than 50 amino acid residues connected one to the other as in a polypeptide.

Fusion protein: refers to a polypeptide containing at

least two different polypeptide domains operatively linked by a typical peptide bond ("fused"), where the two domains correspond to peptides no found fused in nature.

Synthetic peptide: refers to a chemically produced chain of amino acid residues linked together by peptide bonds that is free of naturally occurring proteins and fragments thereof.

B. General Considerations

Vascular permeability (VP) modulation by providing either active or inactive Src or Yes proteins for potentiating or inhibiting angiogenesis, respectively, has been described in co-pending U.S. Patent Application 09/470,881, filed Dec. 22, 1999.

The present invention is directed to the further discovery that vascular leakage and/or edema associated with trauma, disease of injury related increase in vascular permeability can be specifically modulated, and ameliorated, by inhibition of Src family tyrosine kinase activity. In particular, it has been discovered that *in vivo* administration of a Src family tyrosine kinase inhibitor decreases tissue damage due to disease- or injury-related increase in vascular permeability that is not associated with cancer or angiogenesis.

While administration of a Src family tyrosine kinase inhibitor modulates VEGF-induced VP increase, the specific inhibition of Src family kinase activity ameliorates damage to surrounding tissues caused by vascular leakage and/or edema, however the Src family kinase signal is activated.

Vascular permeability is implicated in a variety of disease processes independent of any direct association with

angiogenesis. For example, many stroke induced pathologies and damage are caused by the sudden increase in VP due to trauma to the blood vessel, and thus the ability to specifically modulate VP will allow for novel and effective treatments to reduce the adverse effects of stroke.

Examples of tissue associated with disease or injury induced vascular leakage and/or edema that will benefit from the specific inhibitory modulation using a Src family kinase inhibitor include rheumatoid arthritis, diabetic retinopathy, inflammatory diseases, restenosis, and the like.

Trauma to the head or spine, and other cerebrovascular accident typically caused by ischemic or hemorrhagic events, are a major cause of neurological disorder and related injury. Brain edema or vascular leakage resulting from such injuries, is a life-threatening pathology which triggers systemic and disseminated damage to the brain and spinal cord (central nervous system; CNS) and the ability to specifically modulate the tissue damaging effects of vascular leakage and edema in such instances is very useful.

CNS infections, meningitis, cerebritis, encephalitis, can all result in the adverse pathology including cerebral edema. Treatment of the underlying infection can be supplemented with specific therapy to reduce vascular leakage or edema.

It has been reported that systemic neutralization of VEGF protein using a VEGF receptor IgG fusion protein reduces infarct size following cerebral ischemia, this effect was attributed to the reduction of VEGF-mediated vascular permeability. N. van Bruggen et al., J. Clin. Inves. 104:1613-1620 (1999). However, VEGF is not the critical mediator of vascular permeability increase that Src has now been

discovered to be.

Other diseases or conditions where Src mediated increase in vascular permeability is involved and are thus suitable targets for treatment by the methods and with the compositions of the present invention may include: cerebral hemorrhage, brain and spinal trauma, hypoxia-induced brain and spinal injury; inflammatory disorders of the CNS: viral or bacterial infections (e.g. meningitis, HIV encephalopathy), autoimmune disorders (e.g. multiple sclerosis); diseases with a chronic increase in blood brain barrier permeability (e.g. Morbus Alzheimer); in surgeries where a temporary impairment of perfusion or oxygenation of tissue is needed, as a protective agent; adult respiratory distress syndrome (ARDS); rheumatoid arthritis; and diabetic retinopathy.

C. Src Family Tyrosine kinase Proteins

The terms "Src protein" or "Src" are used to refer collectively to the various forms of tyrosine kinase Src protein described herein, either in active or inactive forms. The terms "Yes protein" or "Yes" are used to refer collectively to the various forms of tyrosine kinase Yes protein described herein, either in active or inactive forms. Also, in the context of the description, reference is also made to Src or Yes encoding nucleic acid genetic sequence or genes. The term "Src family" refers to the group of tyrosine kinases which are related in function and amino acid sequence to Src.

An "inactive Src protein" refers to any of a variety of forms of Src protein which inhibit angiogenesis or VP. An "inactive Yes protein" refers to any of a variety of forms of

Yes protein which inhibit VP. Assays to measure inhibition of VP increase are described herein, and are not to be construed as limiting. A Src protein is considered inactive if the level of angiogenesis is at least 10% lower, preferably 25% lower, and more preferably 50% lower than a control level where no exogenous Src is added to the assay system.

A Src or Yes protein is considered inactive if the level of VP is at least the same as, or 10% lower, preferably 25% lower, and more preferably 50% lower than a control level where no exogenous Src or Yes is added to the assay system.

A preferred assay for measuring inhibition of VP is the Miles assay using Evan's blue dye in mice as described in the Examples, in which VP is measured by the amount of Evan's blue dye leaked from blood vessels.

A preferred inactive Src or Yes protein exhibits reduced tyrosine kinase activity as well. Exemplary inactive Src proteins are described in the Examples, and include Src-251 and Src K295M.

A Src protein useful in the present invention can be produced in any of a variety of methods including isolation from natural sources including tissue, production by recombinant DNA expression and purification, and the like. Src and/or Yes protein can also be provided "in situ" by introduction of a gene therapy system to the tissue of interest which then expresses the protein in the tissue.

A gene encoding a Src or Yes protein can be prepared by a variety of methods known in the art, and the invention is not to be construed as limiting in this regard. For example, the natural history of Src is well known to include a variety of homologs from mammalian, avian, viral and the like species,

and the gene can readily be cloned using cDNA cloning methods from any tissue expressing the protein. A preferred Src for use in the invention is a cellular protein, such as the mammalian or avian homologs designated c-Src. Particularly preferred is human c-Src. A preferred Yes for use in the invention is a human cellular protein, c-Yes. Particularly preferred is human c-Yes-1 encoding for the amino acid sequence as depicted in Figure 8. The protein Yes-1 of Figure 8 is encoded for by a segment of the nucleic acid sequence depicted in Figure 9, and identified as the coding domain segment.

D. Recombinant DNA Molecules and Expression Systems for Expression of Src, Yes, or CSK Protein

The invention describes several nucleotide sequences of particular use in the present invention. These sequences include sequences which encode a Src protein useful in the invention, and various DNA segments, recombinant DNA (rDNA) molecules and vectors constructed for expression of Src protein. These sequences also include sequences which encode a Yes protein useful in the invention, and various DNA segments, recombinant DNA (rDNA) molecules and vectors constructed for expression of Yes protein.

DNA molecules (segments) of this invention therefore can comprise sequences which encode whole structural genes, fragments of structural genes, or combination of genes, and transcription units as described further herein.

A preferred DNA segment is a nucleotide sequence which encodes a Src or Yes protein, or both as defined herein, or fragment thereof. The amino acid residue sequence and

nucleotide sequence of preferred Src and Yes is described in the Examples.

A preferred DNA segment codes for an amino acid residue sequence substantially the same as, and preferably consisting essentially of, an amino acid residue sequence or portions thereof corresponding to a Src or Yes protein described herein. Representative and preferred DNA segments are further described in the Examples.

The amino acid residue sequence of a protein or polypeptide is directly related via the genetic code to the deoxyribonucleic acid (DNA) sequence of the structural gene that codes for the protein. Thus, a structural gene or DNA segment can be defined in terms of the amino acid residue sequence, i.e., protein or polypeptide, for which it codes.

An important and well known feature of the genetic code is its redundancy. That is, for most of the amino acids used to make proteins, more than one coding nucleotide triplet (codon) can code for or designate a particular amino acid residue. Therefore, a number of different nucleotide sequences may code for a particular amino acid residue sequence. Such nucleotide sequences are considered functionally equivalent since they can result in the production of the same amino acid residue sequence in all organisms. Occasionally, a methylated variant of a purine or pyrimidine may be incorporated into a given nucleotide sequence. However, such methylation do not affect the coding relationship in any way.

A nucleic acid is any polynucleotide or nucleic acid fragment, whether it be a polyribonucleotide of polydeoxyribonucleotide, i.e., RNA or DNA, or analogs thereof.

In preferred embodiments, a nucleic acid molecule is in the form of a segment of duplex DNA, i.e, a DNA segment, although for certain molecular biological methodologies, single-stranded DNA or RNA is preferred.

DNA segments are produced by a number of means including chemical synthesis methods and recombinant approaches, preferably by cloning or by polymerase chain reaction (PCR). DNA segments that encode portions of a protein can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al, (1981, J. Am. Chem. Soc., 103:3185-3191), or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define the DNA segment, followed by hybridization and ligation of oligonucleotides to build the complete segment. Alternative methods include isolation of a preferred DNA segment by PCR with a pair of oligonucleotide primers used on a cDNA library believed to contain members which encode a desired protein.

Of course, through chemical synthesis, any desired modifications can be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence. This method is well known, and can be readily applied to the production of the various different modified Src proteins described herein.

Furthermore, DNA segments consisting essentially of structural genes encoding a Src, Yes, or CSK protein can be subsequently modified, as by site-directed or random mutagenesis, to introduce any desired substitutions.

1. Cloning a src or yes Gene

A src or yes gene of this invention can be cloned from a suitable source of genomic DNA or messenger RNA (mRNA) by a variety of biochemical methods. Cloning these genes can be conducted according to the general methods described in the Examples and as known in the art.

Sources of nucleic acids for cloning a src or yes gene suitable for use in the methods of this invention can include genomic DNA or messenger RNA (mRNA) in the form of a cDNA library, from a tissue believed to express these proteins. A preferred tissue is human lung tissue, although any other suitable tissue may be used.

A preferred cloning method involves the preparation of a cDNA library using standard methods, and isolating the Src-encoding, or Yes-encoding nucleotide sequence by PCR amplification using paired oligonucleotide primers based on the nucleotide sequences described herein. Alternatively, the desired cDNA clones can be identified and isolated from a cDNA or genomic library by conventional nucleic acid hybridization methods using a hybridization probe based on the nucleic acid sequences described herein. Other methods of isolating and cloning suitable Src or Yes encoding nucleic acids are readily apparent to one skilled in the art.

2. Gene Transfer and/or Expression Vectors

The invention contemplates a recombinant DNA molecule (rDNA) containing a DNA segment encoding one or more of Src, Yes, or CSK as described herein. An expressible rDNA can be produced by operatively (in frame, expressible) linking a vector to a Src, Yes or CSK encoding DNA segment. Thus, a

recombinant DNA molecule is a hybrid DNA molecule comprising at least two nucleic acids of a nucleotide sequences not normally found together in nature.

The choice of vector to which a DNA segment of the present invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. Typical considerations in the art of constructing recombinant DNA molecules. A vector contemplated by the present invention is at least capable of directing the replication, and preferably also expression, of a structural gene included in the vector DNA segments, to which it is operatively linked.

Where an expression vector contains more than one expressible nucleic acid sequence encoding for desired protein, each of the genes may be regulated by the same regulatory elements upstream of the first gene, or each individually regulated by separate regulatory elements.

Both prokaryotic and eukaryotic expression vectors are familiar to one of ordinary skill in the art of vector construction, and are described by Ausebel, et al., in Current Protocols in Molecular Biology, Wiley and Sons, New York (1993) and by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, (1989). These references also describe many of the general recombinant DNA methods referred to herein.

In one embodiment, a vector contemplated by the present invention includes a procaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule

extrachromosomally in a procaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, those embodiments that include a procaryotic replicon also include a gene whose expression confers drug resistance to a bacterial host transformed therewith. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Those vectors that include a procaryotic replicon can also include a procaryotic promoter capable of directing the expression (transcription and translation) of a structural gene in a bacterial host cell, such as E. coli, transformed therewith. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA), pRSET available from Invitrogen (San Diego, CA) and pPL and pKK223 available from Pharmacia, Piscataway, N.J.

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to form the recombinant DNA molecules of the present invention. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia),

pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), pRc/CMV (Invitrogen, Inc.), the preferred vector described in the Examples, and the like eukaryotic expression vectors.

A particularly preferred system for gene expression in the context of this invention includes a gene delivery component, that is, the ability to deliver the gene to the tissue of interest. Suitable vectors are "infectious" vectors such as recombinant DNA viruses, adenovirus or retrovirus vectors which are engineered to express the desired protein and have features which allow infection of preselected target tissues. Particularly preferred is the replication competent avian sarcoma virus (RCAS) described herein.

Mammalian cell systems that utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, the coding sequence of a polypeptide may be ligated to an adenovirus transcription/ translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted into the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the polypeptide in infected hosts (e.g., see Logan et al., 1984, Proc. Natl. Acad. Sci., USA, 81:3655-3659). Alternatively, the vaccinia virus 7.5K promoter may be used. (e.g., see, Mackett et al., 1982, Proc. Natl. Acad. Sci., USA, 79:7415-7419); Mackett et al., 1984, J. Virol., 49:857-864); Panicali et al., 1982, Proc. Natl. Acad. Sci., USA, 79:4927-4931). Of particular interest are vectors based on

bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver et al., 1981, Mol. Cell. Biol., 1:486). Shortly after entry of this DNA into target cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the neo gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the polypeptide-encoding nucleotide sequence in host cells (Cone et al., 1984, Proc. Natl. Acad. Sci., USA, 81:6349-6353). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionine IIA promoter and heat shock promoters.

Recently, long-term survival of cytomegalovirus (CMV) promoter versus Rous sarcoma virus (RSV) promotor-driven thymidine kinase (TK) gene therapy in nude mice bearing human ovarian cancer has been studied. Cell killing efficacy of adenovirus-mediated CMV promoter-driven herpes simplex virus TK gene therapy was found to be 2 to 10 time more effective than RSV driven therapy. (Tong et al., 1999, Hybridoma 18(1):93-97). The design of chimeric promoters for gene therapy applications, which call for low level expression followed by inducible high-level expression has also been described. (Suzuki et al., 1996, Human Gene Therapy 7:1883-1893).

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using

expression vectors which contain viral origins of replication, host cells can be transformed with a cDNA controlled by appropriate expression control elements (e.g., promoter and enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. As mentioned above, the selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, Cell, 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al, 1962, Proc. Natl. Acad. Sci., USA, 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell, 22:817) genes, which can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells respectively. Also, antimetabolite resistance-conferring genes can be used as the basis of selection; for example, the genes for dhfr, which confers resistance to methotrexate (Wigler et al., 1980, Proc. Natl. Acad. Sci., USA, 77:3567); O'Hare et al., 1981, Proc. Natl. Acad. Sci., USA, 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan et al, 1981, Proc. Natl. Acad. Sci., USA, 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al, 1981, J. Mol. Biol., 150:1); and hygro, which confers resistance to hygromycin (Santerre et al, 1984, Gene, 30:147). Recently, additional selectable genes

have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman et al, 1988, Proc. Natl. Acad. Sci., USA, 85:804); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed., (1987)).

The principal vectors contemplated for human gene therapy, are derived from retroviral origin. (Wilson, 1997, Clin. Exp. Immunol. 107(Sup. 1):31-32; Bank et al., 1996, Bioessays 18(12):999-1007; Robbins et al., 1998, Pharmacol. Ther. 80(1):35-47). The therapeutic potential of gene transfer and antisense therapy has stimulated the development of many vector systems for treating a variety of tissues. (vasculature, Stephan et al., 1997, Fundam. Clin. Pharmacol. 11(2):97-110; Feldman et al., 1997, Cardiovasc. Res. 35(3):391-404; Vassalli et al., 1997, Cardiovasc. Res. 35(3):459-69; Baek et al., 1998, Circ. Res. 82(3):295-305; kidney, Lien et al., 1997, Kidney Int. Suppl. 61:S85-8; liver, Ferry et al., 1998, Hum Gene Ther. 9(14):1975-81; muscle, Marshall et al., 1998, Curr. Opn. Genet. Dev. 8(3):360-5). In addition to these tissues, a critical target for human gene therapy is cancer, either the tumor itself, or associated tissues. (Runnebaum, 1997, Anticancer Res. 17(4B):2887-90; Spear et al., 1998, J. Neurovirol. 4(2):133-47).

Specific examples of viral gene therapy vector systems readily adaptable for use in the methods of the present invention are briefly described below. Retroviral gene

delivery has been recently reviewed by Federspiel and Hughes (1998, Methods in Cell Biol. 52:179-214) which describes in particular, the avian leukosis virus (ALV) retrovirus family (Federspiel et al., 1996, Proc. Natl. Acad. Sci., USA, 93: 4931; Federspiel et al., 1994, Proc. Natl. Acad. Sci., USA, 91: 11241). Retroviral vectors, including ALV and murine leukemia virus (MLV) are further described by Svoboda (1998, Gene 206:153-163).

Modified retroviral/adenoviral expression systems can be readily adapted for practice of the methods of the present invention. For example, murine leukemia virus (MLV) systems are reviewed by Karavanas et al., 1998, Crit. Rev. in Oncology/Hematology 28:7-30. Adenovirus expression systems are reviewed by Von Seggern and Nemerow in Gene Expression Systems (ed. Fernandez & Hoeffler, Academic Press, San Diego, CA, 1999, chapter 5, pages 112-157).

Protein expression systems have been demonstrated to have effective use both in vivo and in vitro. For example, efficient gene transfer to human squamous cell carcinomas by a herpes simplex virus (HSV) type 1 amplicon vector has been described. (Carew et al., 1998, Am. J. Surg. 176:404-408). Herpes simplex virus has been used for gene transfer to the nervous system. (Goins et al., 1997, J. Neurovirol. 3 (Sup. 1):S80-8). Targeted suicide vectors using HSV-TK has been tested on solid tumors. (Smiley et al., 1997, Hum. Gene Ther. 8(8):965-77). Herpes simplex virus type 1 vector has been used for cancer gene therapy on colon carcinoma cells. (Yoon et al., 1998, Ann. Surg. 228(3):366-74). Hybrid vectors have been developed to extend the length of time of transfection, including HSV/AAV (adeno-associated virus) hybrids for

treating hepatocytes. (Fraefel et al., 1997, Mol. Med. 3(12):813-825).

Vaccinia virus has been developed for human gene therapy because of its large genome. (Peplinski et al., 1998, Surg. Oncol. Clin. N. Am. 7(3):575-88). Thymidine kinase-deleted vaccinia virus expressing purine nucleoside pyrophosphorylase has been described for use as a tumor directed gene therapy vector. (Puhlman et al., 1999, Human Gene Therapy 10:649-657).

Adeno-associated virus 2 (AAV) has been described for use in human gene therapy, however AAV requires a helper virus (such as adenovirus or herpes virus) for optimal replication and packaging in mammalian cells. (Snoeck et al., 1997, Exp. Nephrol. 5(6):514-20; Rabinowitz et al., 1998, Curr. Opn. Biotechnol. 9(5):470-5). However, in vitro packaging of an infectious recombinant AAV has been described, making this system much more promising. (Ding et al., 1997, Gene Therapy 4:1167-1172). It has been shown that the AAV mediated transfer of ecotropic retrovirus receptor cDNA allows ecotropic retroviral transduction of established and primary human cells. (Qing et al., 1997, J. Virology 71(7):5663-5667). Cancer gene therapy using an AAV vector expressing human wild-type p53 has been demonstrated. (Qazilbash et al., 1997, Gene Therapy 4:675-682). Gene transfer into vascular cells using AAV vectors has also been shown. (Maeda et al., 1997, Cardiovascular Res. 35:514-521). AAV has been demonstrated as a suitable vector for liver directed gene therapy. (Xiao et al., 1998, J. Virol. 72(12):10222-6). AAV vectors have been demonstrated for use in gene therapy of brain tissues and the central nervous system. (Chamberlin et al., 1998, Brain Res. 793(1-2):169-75; During et al., 1998, Gene Therapy 5(6):820-

7). AAV vectors have also been compared with adenovirus vectors (AdV) for gene therapy of the lung and transfer to human cystic fibrosis epithelial cells. (Teramoto et al., 1998, J. Virol. 72(11):8904-12).

Chimeric AdV/retroviral gene therapy vector systems which incorporate the useful qualities of each virus to create a nonintegrative AdV that is rendered functionally integrative via the intermediate generation of a retroviral producer cell. (Feng et al., 1997, Nat. Biotechnology 15(9):866-70; Bilbao et al., 1997, FASEB J 11(8):624-34). This powerful new generation of gene therapy vector has been adapted for targeted cancer gene therapy. (Bilbao et al., 1998, Adv. Exp. Med. Biol. 451:365-74). Single injection of AdV expressing p53 inhibited growth of subcutaneous tumor nodules of human prostrate cancer cells. (Asgari et al., 1997, Int. J. Cancer 71(3):377-82). AdV mediated gene transfer of wild-type p53 in patients with advanced non-small cell lung cancer has been described. (Schuler et al., 1998, Human Gene Therapy 9:2075-2082). This same cancer has been the subject of p53 gene replacement therapy mediated by AdV vectors. (Roth et al., 1998, Semin. Oncol. 25(3 Suppl 8):33-7). AdV mediated gene transfer of p53 inhibits endothelial cell differentiation and angiogenesis in vivo. (Riccioni et al., 1998, Gene Ther. 5(6):747-54). Adenovirus-mediated expression of melanoma antigen gp75 as immunotherapy for metastatic melanoma has also been described. (Hirschowitz et al., 1998, Gene Therapy 5:975-983). AdV facilitates infection of human cells with ecotropic retrovirus and increases efficiency of retroviral infection. (Scott-Taylor, et al., 1998, Gene Ther. 5(5):621-9). AdV vectors have been used for gene transfer to vascular smooth muscle cells

(Li et al., 1997, Chin. Med. J.(Engl) 110(12):950-4), squamous cell carcinoma cells (Goebel et al., 1998, Otolarynol Head Neck Surg 119(4):331-6), esophageal cancer cells (Senmaru et al., 1998, Int J. Cancer 78(3):366-71), mesangial cells (Nahman et al., 1998, J. Investig. Med. 46(5):204-9), glial cells (Chen et al., 1998, Cancer Res. 58(16):3504-7), and to the joints of animals (Ikeda et al., 1998, J. Rheumatol. 25(9):1666-73). More recently, catheter-based pericardial gene transfer mediated by AcV vectors has been demonstrated. (March et al., 1999, Clin. Cardiol. 22(1 Suppl 1):I23-9). Manipulation of the AdV system with the proper controlling genetic elements allows for the AdV-mediated regulable target gene expression in vivo. (Burcin et al., 1999, PNAS (USA) 96(2):355-60).

Alphavirus vectors have been developed for human gene therapy applications, with packaging cell lines suitable for transformation with expression cassettes suitable for use with Sindbis virus and Semliki Forest virus-derived vectors. (Polo et al., 1999, Proc. Natl. Acad. Sci., USA, 96:4598-4603). Noncytopathic flavivirus replicon RNA-based systems have also been developed. (Varnavski et al., 1999, Virology 255(2):366-75). Suicide HSV-TK gene containing sinbis virus vectors have been used for cell-specific targeting into tumor cells. (Iijima et al., 1998, Int. J. Cancer 80(1):110-8).

Retroviral vectors based on human foamy virus (HFV) also show promise as gene therapy vectors. (Trobridge et al., 1998, Human Gene Therapy 9:2517-2525). Foamy virus vectors have been designed for suicide gene therapy. (Nestler et al., 1997, Gene Ther. 4(11):1270-7). Recombinant murine cytomegalovirus and promoter systems have also been used as vectors for high level

expression. (Manning et al., 1998, J. Virol. Meth. 73(1):31-9; Tong et al., 1998, Hybridoma 18(1):93-7).

Gene delivery into non-dividing cells has been made feasible by the generation of Sendai virus based vectors. (Nakanishi et al., 1998, J. Controlled Release 54(1):61-8).

In other efforts to enable the transformation of non-dividing somatic cells, lentiviral vectors have been explored. Gene therapy of cystic fibrosis using a replication-defective human immunodeficiency virus (HIV) based vector has been described. (Goldman et al., 1997, Human Gene Therapy 8:2261-2268). Sustained expression of genes delivered into liver and muscle by lentiviral vectors has also been shown. (Kafri et al., 1997, Nat. Genet. 17(3):314-7). However, safety concerns are predominant, and improved vector development is proceeding rapidly. (Kim et al., 1998, J. Virol. 72(2):994-1004). Examination of the HIV LTR and Tat yield important information about the organization of the genome for developing vectors. (Sadaie et al., 1998, J. Med. Virol. 54(2):118-28). Thus the genetic requirements for an effective HIV based vector are now better understood. (Gasmi et al., 1999, J. Virol. 73(3):1828-34). Self inactivating vectors, or conditional packaging cell lines have been described. (for example Zuffery et al., 1998, J. Virol. 72(12):9873-80; Miyoshi et al., 1998, J. Virol. 72(10):8150-7; Dull et al., 1998, J. Virol. 72(11):8463-71; and Kaul et al., 1998, Virology 249(1):167-74). Efficient transduction of human lymphocytes and CD34+ cells by HIV vectors has been shown. (Douglas et al., 1999, Hum. Gene Ther. 10(6):935-45; Miyoshi et al., 1999, Science 283(5402):682-6). Efficient transduction of nondividing human cells by feline immunodeficiency virus (FIV) lentiviral vectors has been

described, which minimizes safety concerns with using HIV based vectors. (Poeschla et al., 1998, Nature Medicine 4(3):354-357). Productive infection of human blood mononuclear cells by FIV vectors has been shown. (Johnston et al., 1999, J. Virol. 73(3):2491-8).

While many viral vectors are difficult to handle, and capacity for inserted DNA limited, these limitations and disadvantages have been addressed. For example, in addition to simplified viral packaging cell lines, Mini- viral vectors, derived from human herpes virus, herpes simplex virus type 1 (HSV-1), and Epstein-Barr virus (EBV), have been developed to simplify manipulation of genetic material and generation of viral vectors. (Wang et al., 1996, J. Virology 70(12):8422-8430). Adaptor plasmids have been previously shown to simplify insertion of foreign DNA into helper-independent Retroviral vectors. (1987, J. Virology 61(10):3004-3012).

Viral vectors are not the only means for effecting gene therapy, as several non-viral vectors have also been described. A targeted non-viral gene delivery vector based on the use of Epidermal Growth Factor/DNA polyplex (EGF/DNA) has been shown to result in efficient and specific gene delivery. (Cristiano, 1998, Anticancer Res. 18:3241-3246). Gene therapy of the vasculature and CNS have been demonstrated using cationic liposomes. (Yang et al., 1997, J. Neurotrauma 14(5):281-97). Transient gene therapy of pancreatitis has also been accomplished using cationic liposomes. (Denham et al., 1998, Ann. Surg. 227(6):812-20). A chitosan-based vector/DNA complexes for gene delivery have been shown to be effective. (Erbacher et al., 1998, Pharm. Res. 15(9):1332-9). A non-viral DNA delivery vector based on a terplex system has been

described. (Kim et al., 1998, 53(1-3):175-82). Virus particle coated liposome complexes have also been used to effect gene transfer. (Hirai et al., 1997, Biochem. Biophys. Res. Commun. 241(1):112-8).

Gene therapy by direct tumor injections of nonviral T7 vector encoding a thymidine kinase gene has been demonstrated. (Chen et al., 1998, Human Gene Therapy 9:729-736). Plasmid DNA preparation is important for direct injection gene transfer. (Horn et al., 1995, Hum. Gene Ther. 6(5):656-73). Modified plasmid vectors have been adapted specifically for direct injection. (Hartikka et al., 1996, Hum. Gene Ther. 7(10):1205-17).

Thus, a wide variety of gene transfer/gene therapy vectors and constructs are known in the art. These vectors are readily adapted for use in the methods of the present invention. By the appropriate manipulation using recombinant DNA/molecular biology techniques to insert an operatively linked Src or yes, or both (either active or inactive) into the selected expression/delivery vector, many equivalent vectors for the practice of the present invention can be generated.

E. Methods and Compositions For Modulation of Vascular Permeability

The invention provides for a method for the specific modulation of VP of blood vessels in a tissue associated with an injury, disease or trauma, and thereby ameliorate damage to the tissue due to vascular leakage and/or edema. In particular, the present invention describes methods for inhibiting injury related increases in VP which can result in

tissue damage due to vascular leakage and/or edema. The present invention is most particularly directed towards ameliorating tissue damage due to vascular leakage or edema associated with injury, trauma or disease using a Src family tyrosine kinase inhibitor, where the increase in VP is either VEGF-induced or otherwise induced.

Generally, the method of the invention comprises administering to a tissue associated with a disease process or blood vessel injury or trauma condition, a composition comprising a Src family tyrosine kinase inhibitor. A Src family tyrosine kinase inhibitor can be a chemical Src inhibitor, a protein Src inhibitor, or a nucleic acid Src inhibitor.

Examples of suitable chemical Src family tyrosine kinase inhibitors include and are not limited to PP1, PP2, PD173955, AGL1872, PD162531, Radicicol R2146, Geldanamycin and the like.

PP1 (from Biomol, by license from Pfizer), was the synthetic Src inhibitor used for these studies. PP1 is part of the pyrazolopyrimidine family of Src inhibitors. Other synthetic Src inhibitors include PP2 (from Calbiochem, on license form Pfizer) which is related in structure to PP1 and has also been shown to block Src family kinase activity. (Hanke et al., 1996, J. Biol. Chem. 271(2): 695-701). Other specific Src kinase inhibitors include PD173955 (Moasser et al., 1999, Cancer Res. 59:6145-6152; Parke Davis) for which the structure has been published. PD162531 (Owens et al., 2000, Mol. Biol. Cell 11:51-64) is also a specific Src kinase inhibitor from Parke Davis but the structure is not accessible from the literature. Geldanamycin is also a Src kinase inhibitor, available from Life Technologies. Radicicol, which is offered commercially by

different companies (e.g. Calbiochem, RBI, Sigma), is an antifungal macrocyclic lactone antibiotic that also acts as an unspecific protein tyrosine kinase inhibitor and was shown to inhibit Src kinase activity. Preferred chemical inhibitors are PP1 and PP2 or the like, a most preferred chemical inhibitor being PP1.

Additional suitable Src family tyrosine kinase inhibitors can be identified and characterized using standard assays known in the art. For example screening of chemical compounds for potent and selective inhibitors for Src or other tyrosine kinases has been done and have resulted in the identification of chemical moieties useful in potent inhibitors of Src family tyrosine kinases.

For example, catechols have been identified as important binding elements for a number of tyrosine kinase inhibitors derived from natural products, and have been found in compounds selected by combinatorial target-guided selection for selective inhibitors of c-Src. Maly, D.J., et al. (2000, "Combinatorial target-guided ligand assembly: Identification of potent subtype-selective c-Src inhibitors" PNAS(USA) 97(6): 2419-2424). Combinatorial chemistry based screening of candidate inhibitor compounds, using moieties known to be important to Src inhibition as a starting point, is a potent and effective means for isolating and characterizing other chemical inhibitors of Src family tyrosine kinases.

However, even careful selection of potential binding elements based upon the potential for mimicking a wide range of functionalities present on polypeptides and nucleic acids can be used to perform combinatorial screens for active inhibitors. For example, O-methyl oxime libraries are particularly suited for

this task, given that the library is easily prepared by condensation of O-methylhydroxylamine with any of a large number of commercially available aldehydes. O-alkyl oxime formation is compatible with a wide range of functionalities which are stable at physiological pH. Malay et al., *supra*.

As described, suitable Src family kinase inhibitors also include VP-inhibiting amount of an inactive Src or Yes protein, or mixture thereof, or nucleic acid vector expressing inactive Src or Yes, or both, according to the methods of this invention.

Other suitable Src family kinase inhibitors include CSK, or nucleic acid vector expressing inactivating amounts of CSK, according to the methods of this invention.

As described herein, any of a variety of tissues, or organs comprised of organized tissues, can be a location for VP in disease conditions including brain, skin, muscle, gut, connective tissue, joints, bones and the like tissue in which blood vessels are present.

The patient that can be treated by a method embodying the present invention is desirably a human patient, although it is to be understood that the principles of the invention indicate that the present methods are effective with respect to all mammals. Accordingly, included in the term "patient" as used herein. In this context, a mammal is understood to include any mammalian species in which treatment of vascular leakage or edema associated tissue damage is desirable, particularly agricultural and domestic mammalian species.

A method embodying this invention comprises administering to a mammalian patient a therapeutically effective amount of a physiologically tolerable composition containing a chemical Src family tyrosine kinase inhibitor, an inactive Src or Yes

protein, active CSK protein, a nucleic acid encoding for such protein, or mixtures thereof, in practicing the methods of the invention.

The dosage ranges for the administration of chemical Src family tyrosine kinase inhibitors, such as PP1 can be in the range of about .1 mg/kg body weight to about 10 mg/kg body weight, or the limit of solubility of the active agent in the pharmaceutical carrier. Preferably, typical dosages can be from about 1 mg/kg body weight to about 9 mg/kg body weight. Lower dosages, such as from .1 mg/kg body weight to about 1 mg/kg body weight can be optimized for multiple administration to treat chronic conditions. Typical dosages for treating acute conditions that are less severe, easily accessible, and where the route of administration is more direct, can be from about 1 mg/kg body weight to about 3 mg/kg body weight. Depending upon the severity of the injury, location, or the route of administration, a higher dose of from about 3 mg/kg body weight to 10 mg/kg body weight (or limit of solubility of the agent in the pharmaceutical carrier) may be used when encountering a more severe injury, hard to access location, or where administration can only be via indirect systemic route.

In the case of acute injury or trauma, it is best to administer treatment as soon as possible after the occurrence of the incident. However, time for effective administration of a Src family tyrosine kinase inhibitors can be within about 48 hours of the onset of injury or trauma, in the case of acute incidents. It is preferred that administration occur within about 24 hours of onset, within 12 hours being better, and most preferred that administration take place within about 6 hours of onset. Administration after 48 hours of initial injury may be

appropriate to ameliorate additional tissue damage due to further vascular leakage or edema, however the effect on the initial tissue damage may be greatly reduced.

Where prophylactic administration is made to prevent vascular leakage or edema associated with surgical procedure, or made in view of predisposing diagnostic criteria, administration can occur prior to any actual VP increase, or during such VP increase causing event. For the treatment of chronic conditions which lead to VP increase and associated vascular leaking or edema, administration of active Src family tyrosine kinase inhibitors can be made with a continuous dosing regimen.

The dosage ranges for the administration of an inactive Src or Yes protein, or active CSK protein depend upon the form of the protein, and its potency, as described further herein, and are amounts large enough to produce the desired effect in which VP and the disease symptoms mediated by VP are ameliorated. The dosage should not be so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like.

A therapeutically effective VP modulating amount is an amount of active CSK or inactive Src or Yes protein, or mixture thereof, or nucleic acid encoding such protein, sufficient to produce a measurable modulation of VP in the tissue being treated, ie., a VP-modulating amount. Modulation of VP can be measured by assay as described herein, or by other methods known to one skilled in the art. Modulation of VP can be measured by the Miller assay, as described herein, or by other methods known to one of skill in the art.

Generally, the dosage can vary with the age, condition, sex and extent of the disease in the patient and can be determined

by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication.

The pharmaceutical compositions of the invention can be administered parenterally by injection or by gradual infusion over time. Although the tissue to be treated can typically be accessed in the body by systemic administration and therefore most often treated by intravenous administration of therapeutic compositions, other tissues and delivery means are contemplated where there is a likelihood that the tissue targeted contains the target molecule. Thus, compositions of the invention can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, and can be delivered by peristaltic means.

Intravenous administration is effected by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

In one preferred embodiment the active agent is administered in a single dosage intravenously. Localized administration can be accomplished by direct injection or by taking advantage of anatomically isolated compartments, isolating the microcirculation of target organ systems, reperfusion in a circulating system, or catheter based temporary occlusion of target regions of vasculature associated with diseased tissues.

The compositions are administered in a manner compatible

with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered and timing depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient to be administered depend on the judgement of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for administration are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for in vivo therapies are contemplated.

The methods of the invention ameliorating tissue damage due to vascular leakage or edema associated with a disease condition, injury or trauma ameliorates symptoms of the disease and, depending upon the disease, can contribute to cure of the disease. The extent of vascular permeability in a tissue, and therefore the extent of inhibition achieved by the present methods, can be evaluated by a variety of methods. In particular, the methods are suitable for ameliorating stroke or other cerebrovascular accident related injury to the CNS that occur due to injury induced increase of VP, and subsequent vascular leakage and/or edema damage to associated tissues.

In one related embodiment, a tissue to be treated is an inflamed tissue and the vascular permeability to be inhibited is due to VEGF mediated stimulation. For this type of affliction, the method contemplates inhibition of VP in arthritic tissues,

such as in a patient with chronic articular rheumatism, in immune or non-immune inflamed tissues, in psoriatic tissue, and the like.

In another related embodiment, a tissue to be treated is a retinal tissue of a patient with a retinal disease such as diabetic retinopathy, macular degeneration or neovascular glaucoma and the VP to be inhibited is retinal tissue VP where there is neovascularization of retinal tissue.

The present method for inhibiting vascular permeability in a tissue associated with a injury or disease condition, and therefore for also practicing the methods for treatment of vascular permeability-related diseases, comprises contacting a tissue in which increased vascular permeability is occurring, or is at risk for occurring, with a composition comprising a therapeutically effective amount of a Src family tyrosine kinase inhibitor.

Modulation of VP, and amelioration of tissue damage due to vascular leakage and edema can occur within a short time after administration of the therapeutic composition. Most therapeutic effects can be visualized within 3 days of administration, in the case of acute injury or trauma. Typically, effects of chronic administration will not be as readily apparent.

The time-limiting factors include rate of tissue absorption, cellular uptake, protein translocation or nucleic acid translation (depending on the therapeutic) and protein targeting. Thus, tissue damage modulating effects can occur in as little as an hour from time of administration of the inhibitor. Additional or prolonged exposure to Src family tyrosine kinase inhibitors can also be done, utilizing the proper conditions. Thus, a variety of desired therapeutic time

frames can be designed by modifying such parameters.

F. Therapeutic Compositions

The present invention contemplates therapeutic compositions useful for practicing the therapeutic methods described herein.

Chemical therapeutic compositions of the present invention contain a physiologically tolerable carrier together with a Src family tyrosine kinase inhibitor dissolved or dispersed therein as an active ingredient.

Protein therapeutic compositions of the present invention contain a physiologically tolerable carrier together with an inactive Src, inactive Yes, or active CSK protein dissolved or dispersed therein as a Src family tyrosine kinase inhibitor.

Nucleic acid therapeutic compositions of the present invention contain a physiologically tolerable carrier together with a nucleic acid which encodes for an inactive Src, inactive Yes, or active CSK protein dissolved or dispersed therein as a Src family tyrosine kinase inhibitor.

Suitable Src family tyrosine kinase inhibitors will specifically inhibit the biological tyrosine kinase activity of Src family tyrosine kinases. A most suitable Src family tyrosine kinase will have primary specificity for inhibiting the activity of the ^{pp60}Src protein, and secondarily inhibit the most closely related Src family tyrosine kinases such as Yes. Examples of particularly suitable Src family tyrosine kinase inhibitors include PP1, PP2, PD173955, AGL1872, PD162531, Radicicol R2146, Geldanamycin and the like. Additional suitable chemical Src family tyrosine kinase inhibitors can be identified and characterized using standard assays known in the art.

Mutations in Src shown to be inhibiting VP instead of stimulating it, are referred to as inactive Src mutations. Proteins having mutation that confer this inhibitory activity are also referred to as dominant negative Src proteins in that they inhibit VP, including that resulting from endogenous activity of Src as well as enhanced Src activity resulting from growth factor stimulation. Thus certain mutations of wild type c-Src of the present invention can also function as a dominant negative with respect to their ability to block blood vessel growth and VP, and for example, therefore decrease VP in vivo.

Therefore, other suitable Src family tyrosine kinase inhibitors can include inactive forms of Src and Yes protein that can antagonize Src or Yes activity, resulting in inhibition or decrease in vascular permeability of the blood vessels in the target tissue. A preferred inactive Src protein is Src 251. Another preferred inactive Src protein is Src K295M. A preferred inactive Yes protein will have diminished kinase activity as compared with the wild-type protein.

Other Src family tyrosine kinase inhibitors can be antisense nucleic acids, nucleic acid analogs, or protein nucleic acids which inhibit the expression of Src or Yes genes in targeted cells. The antisense molecules can be a therapeutically effective VP modulating amount when said antisense nucleic acid, capable of hybridizing to the mRNA encoding for Src or Yes protein, can hybridize to such mRNA and result in an inhibition of cell expression of tyrosine kinase protein Src or Yes, when transfected into a target cell in a suitable pharmaceutical carrier.

As described, preferred inhibitory c-Src protein includes the Src 251 in which only the first 251 amino acids of Src are

expressed. This construct lacks the entire kinase domain and is therefore referred to as "kinase dead" Src protein. A second construct is the Src (K295M) mutation in which the lysine amino acid residue 295 is mutated into a methionine. This point mutation in the kinase domain prevents ATP binding and also blocks kinase-dependent Src functions related to vascular cell and tumor cell signaling and proliferation.

With respect to the point mutations, any mutation resulting in the desired inhibitory activity is contemplated for use in this invention. Fusion protein constructs combining the desired Src protein (mutation or fragment thereof) with expressed amino acid tags, antigenic epitopes, fluorescent protein, or other such protein or peptides are also contemplated, so long as the desired modulating effect of the Src protein is intact.

Similarly, addition of an exogenous inhibitor of Src protein activity or the stimulation of expression of such inhibitor within the targeted tissues, such as CSK (C-terminal Src Kinase), is also a means for inhibiting Src activity. Phosphorylation of tyrosine inactivating Src, is a means for negative regulation by the c-terminal Src kinase, referred to as CSK. (Nada et al., 1991, Nature 351: 69-72; Okada et al., 1991, J. Biol. Chem. 266(36): 24249-24252). When CSK phosphorylates aa527 in the wild-type Src, the Src protein is inactivated. Thus, CSK is a useful and potent inhibitor of Src activity. Human CSK protein sequence of 450 amino acids is identified by accession number P41240 and can be found in the swiss protein data base. A human CSK encoding mRNA nucleic acid sequence is identifed by accession number NM 004383 in the GenBank database.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof,

as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as injectable either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified or presented as a liposome composition.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic

bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

A pharmaceutical composition of the invention typically contains a vascular permeability-modulating amount of a Src, Yes and/or CSK protein, or sufficient expression vector to express an effective amount of inactive Src, Yes, or active CSK protein, typically formulated to contain an amount of at least 0.1 weight percent of protein per weight of total pharmaceutical composition. Thus, for example, 0.1 weight percent is 0.1 grams of protein per 100 grams of total composition. For expression vectors, the amount administered depends on the properties of the expression vector, the tissue to be treated, and the like considerations. Thus, an effective amount of a Src family tyrosine kinase inhibitor in a pharmaceutical composition is that amount which results in therapeutically effective

modulation of Src regulated vascular permeability. A therapeutic amount of any pharmaceutical composition is one which, on its own, results in the amelioration of vascular leakage or edema related tissue damage.

G. Article of Manufacture

The invention also contemplates an article of manufacture which is a labelled container for providing a therapeutically effective amount of a Src family tyrosine kinase inhibitor. The inhibitor may be a packaged chemical, protein or nucleic acid Src family tyrosine kinase inhibitor, combinations of more than one, or mixtures thereof. An article of manufacture comprises packaging material and a pharmaceutical agent contained within the packaging material. The article of manufacture may also contain two or more sub-therapeutically effective amounts of a pharmaceutical composition, which together act synergistically to result in amelioration of tissue damage due to vascular leakage or edema.

The pharmaceutical agent in an article of manufacture is any of the compositions of the present invention suitable for providing a Src family tyrosine kinase inhibitor, formulated into a pharmaceutically acceptable form as described herein according to the disclosed indications. Thus, the composition can comprise a chemical inhibitory such as PP1, PP2, PD173955, AGL1872, PD162531, Radicicol R2146, and Geldanamycin, a protein inhibitor such as inactive Src, inactive Yes, active CSK protein, or a nucleic acid molecule which is capable of expressing such protein or combination of proteins. The article of manufacture contains an amount of pharmaceutical agent sufficient for use in treating a condition indicated herein,

either in unit or multiple dosages.

The packaging material comprises a label which indicates the use of the pharmaceutical agent contained therein, e.g., for treating conditions assisted by the inhibition of vascular permeability increase, and the like conditions disclosed herein. The label can further include instructions for use and related information as may be required for marketing. The packaging material can include container(s) for storage of the pharmaceutical agent.

As used herein, the term packaging material refers to a material such as glass, plastic, paper, foil, and the like capable of holding within fixed means a pharmaceutical agent. Thus, for example, the packaging material can be plastic or glass vials, laminated envelopes and the like containers used to contain a pharmaceutical composition including the pharmaceutical agent.

In preferred embodiments, the packaging material includes a label that is a tangible expression describing the contents of the article of manufacture and the use of the pharmaceutical agent contained therein.

Examples

The following examples relating to this invention are illustrative and should not, of course, be construed as specifically limiting the invention. Moreover, such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are to be considered to fall within the scope of the present invention hereinafter claimed.

1. Preparation of c-Src or c-yes Expression Constructs

For preparing the expression constructs useful in modulating VP by the methods of the present invention, c-Src cDNA is manipulated and inserted into an expression construct/vector.

The cDNA sequence encoding for wild-type (i.e., endogenous) chicken c-Src is shown in Figure 1 (SEQ ID NO.:2) with the encoded amino acid residue sequence shown in Figure 2 (SEQ ID NO.:3). The encoded protein sequence is translated from the cDNA nucleotide positions 112 to 1713. The nucleic acid sequence corresponding to the nucleic acid sequence of human c-Src cDNA (SEQ ID NO.:4) and encoded amino acid residue (SEQ ID NO.:5) sequences are shown respectively in Figures 3 and 4. For the human protein sequence, the coding sequence begins at nucleotide position 134 to 1486 of the cDNA.

Wild-type as well as a number of mutated c-Src cDNAs were prepared. Mutated c-Src constructs were prepared by site-directed mutagenesis as described by Kaplan et al., EMBO J., 13:4745-4756 (1994). The mutated c-Src constructs for encoding mutated Src proteins for use in the methods of the present invention are described in Kaplan et al., id.. Kaplan et al. describe various mutated c-Src constructs and encoded proteins of useful for the practice of this invention. For example, Kaplan et al. depict several products of chicken c-Src alleles in their figure 1, including SrcA and Src251.

Two categories of c-Src function to modulate VP, one category contains Src molecules that increase VP and thus are considered to be active proteins. Wild-type Src along with various mutations that retain Src activity to induce VP are catagorized as active Src protein. One mutation of wild type c-

Src which functions in this context with respect to its ability to induce blood vessel growth and VP is the Src A mutant having a point mutation at amino acid (aa) residue position 527 changing tyrosine 527 to phenylalanine. This site is normally a site for negative regulation by the c-terminal Src kinase, referred to as kinase CSK. When CSK phosphorylates aa527 in the wild-type Src, the protein is inactivated. However, in mutated Src A at aa527, the regulatory tyrosine converted to phenylalanine thus conferring upon the protein a constitutively (i.e., permanently) active protein not subject to inactivation by phosphorylation.

Mutations in Src can inhibit VP, and such mutations are referred to as inactive Src mutations. Proteins having mutation that confer this inhibitory activity are also referred to as dominant negative Src proteins in that they inhibit VP, including that resulting from endogenous activity of Src as well as enhanced Src activity resulting from growth factor stimulation. Thus certain mutations of wild type c-Src of the present invention can function as a dominant negative with respect to their ability to decrease VP in vivo.

Such preferred inhibitory c-Src protein includes the Src 251 in which only the first 251 amino acids of Src are expressed. This construct lacks the entire kinase domain and is therefore referred to as "kinase dead" Src protein. A second construct is the Src (K295M) mutation in which the lysine amino acid residue 295 is mutated into a methionine. This point mutation in the kinase domain prevents ATP binding and also blocks kinase-dependent Src functions related to vascular cell and tumor cell signaling and proliferation.

With respect to the point mutations, any mutation resulting

in the desired inhibitory activity is contemplated for use in this invention. Fusion protein constructs combining the desired Src protein (mutation or fragment thereof) with expressed amino acid tags, antigenic epitopes, fluorescent protein, or other such protein or peptides are also contemplated, so long as the desired modulating effect of the Src protein is intact.

Src Family kinase Yes has been previously described, but not much has been known about its cellular function. (Burck et al., 1988, The Oncogenes, Springer-Verlag, New York, pp. 133-155; Marth et al., 1985, Cell, 43:393; Semba et al., 1986, PNAS(USA) 83:5459; Shibuya et al., 1982, J. Virol. 42:143; Yoshida et al., 1985, Jpn. J. Cancer Res. 76:559). Preferred active human Yes protein are encoded for by nucleic acid described in Sukegawa et al. (1987, Mol. Cell Biol. 7:41-47). Inactivating modifications to human Yes protein and nucleic acids encoding Yes can be accomplished as described for Src.

TABLE I

Src/Mutation	Src Function		Effect on VP
c-Src	+	active	stimulates
SrcA (T527F)	+	active	stimulates
Src527(point)	+	active	stimulates
Src251	-	inactive	inhibits
Src (truncate)	-	inactive	inhibits
Src(K295M)	-	inactive	inhibits
Src295 (point)	-	inactive	inhibits

One preferred expression construct for use in the present invention is the RCASBP(A) construct (SEQ ID NO.:1). This expression vector is based on a series of replication competent

avian sarcoma viruses with an enhanced Bryan polymerase (BP) for improved titre, and is specific for the A type envelope glycoprotein expressed on normal avian cells (Reviewed in Methods in Cell Biology, 52:179-214 (1997); see also, Hughes et al., 1987, J. Virol. 61:3004-3012; Fekete & Cepko, 1993, Mol. Cellular Biol. 13(4):2604-2613; Itoh et al., 1996, Development 122:291-300; and Stott et al., 1998, BioTechniques 24:660-666). The complete sequence of RCASBP(A) (SEQ ID NO.:1) is given in the sequence listing, and a restriction map of the construct is depicted as Figure 7, referred to herein as RCAS.

Briefly, cloning of a Src cDNA sequence for expression thereof was accomplished by inserting a linker containing Not I-BstB1-Not I restriction sites into a unique Not I site in the 5' end of Src 251. Src has a unique Cla I site at the 3' end. Digestion of Src 251 with BstB1 and Cla I generated a BstB1-ClaI fragment which was then ligated into the Cla I site on RCASBP(A). A BstB1 overhang allows for ligation with a Cla I overhang that will not be recut with Cla I.

The Src constructs suitable for use in practicing the present invention are readily obtained in the above vector by first digesting the RCAS vector containing Src 251 with Not I and Cla I (in a DAM+ background) to allow for insertion of a similarly digested Src cDNA. Therefore this initial RCASBP(A) construct containing Src 251 was further used to subclone all other Src constructs as described above and in Kaplan et al. (1994, The EMBO J. 13(20):4745-4756), into RCASBP(A) via a Not I-Cla I fragment generated through the Src 251 construction. To produce the desired c-Src mutations in the cDNA, standard site-directed mutagenesis procedures familiar to one of ordinary skill in the art were utilized. PCR primers designed to

incorporate the desired mutations were also designed with restriction sites to facilitate subsequent cloning steps. Entire segments of Src encoding nucleic acid sequences are deleted from the nucleic acid constructs through PCR amplification techniques based on the known cDNA sequences of chicken, human and the like homologs of Src and subsequent formation of new constructs.

In one embodiment of the invention, the 3' PCR primer used to amplify Src nucleic acids also encodes an in-frame sequence. Use of this primer adds a 9E10-myc epitope tag to the carboxyl terminus of the subsequent Src construct.

The following amino acids were added after amino acid 251 of Src to generate vector constructs containing the 9E10-myc epitope tag: VDMEQKLIAEEDLN (SEQ ID NO.: 6). Two separate PCRs were carried out for each construct and similar results were obtained. All mutant constructs constructed by PCR were also sequenced by PCR to confirm predicted DNA sequence of clones. Wild-type and mutated Src cDNAs for use in the expression systems of the present invention are also available from Upstate Biotech Laboratories, Lake Placid, NY which sells avian as well as human Src, and several kinase dead and activated mutated forms.

Alternative expression vectors for use in the expressing the modulatory proteins of the present invention also include adenoviral vectors as described in US Patent Numbers 4,797,368, 5,173,414, 5,436,146, 5,589,377, and 5,670,488. Alternative methods for the delivery of modulatory proteins include delivery of Src, Yes, or CSK cDNA with a non-viral vector system as described in US Patent Number 5,675,954 and delivery of the cDNA itself as naked DNA as described in US Patent Number 5,589,466.

Delivery of constructs of this invention is also not limited to topical application of a viral vector as described in the CAM assay system below. For example, viral vector preparations are also injected intravenously for systemic delivery into the vascular bed. These vectors are also targetable to sites of increased neovascularization by localized injection of a tumor, as an example.

In vitro expressed proteins are also contemplated for delivery thereof following expression and purification of the selected Src protein by methods useful for delivery of proteins or polypeptides. One such method includes liposome delivery systems, such as described in US Patent Numbers 4,356,167, 5,580,575, 5,542,935 and 5,643,599. Other vector and protein delivery systems are well known to those of ordinary skill in the art for use in the expression and/or delivery of the Src, Yes or CSK proteins of the present invention.

a) Activation of Endogenous Src by bFGF or VEGF

To assess the effects of growth factors on Src activity in modulating vascular permeability, the following assays were performed. Tissue extracts of 10 day old chick CAMs that had been exposed to bFGF or VEGF (2 μg/ml) for 2 hours were lysed. Endogenous Src was immunoprecipitated from equivalent amounts of total protein and subjected to an in vitro immune complex kinase assay using a FAK-GST fusion protein as a substrate, electrophoresed and transferred to nitrocellulose.

The results of the assay are shown in Figure 5 where the increase in Src activity is evident in the increased density of the gel with either bFGF or VEGF treatment as compared to untreated (mock) samples that are indicative of baseline Src activity in the CAM assay. Both bFGF and VEGF resulted in

approximately a 2 fold increase of endogenous Src activity present in the CAM. The above kinase assay blot was also probed with an anti-Src antibody as a loading control for equivalent Src and IgG content.

b) Retroviral Expression of Src A Activates Vascular MAP Kinase Phosphorylation

The effect of Src A as compared to growth factors VEGF and PMA on vascular MAP kinase phosphorylation was also assessed following the assay procedures described above and herein. Tissue extracts of 10 day old chick CAMs exposed to VEGF or PMA (another mitogen at a comparable concentration) for 30 minutes were compared to those infected with Src A-expressing retrovirus for 48 hours. Src was than immunoprecipitated from equivalent amounts of total protein extract and subjected to an in vitro immune complex kinase assay using a FAK-GST fusion protein as a substrate, electrophoresed and transferred to nitrocellulose.

The results of this assay are shown in Figure 6A where untreated CAMs (NT) exhibit base-line endogenous Src-mediated vascular MAP kinase phosphorylation. Both VEGF and PMA resulted in an approximate 2 fold increase over baseline. In contrast, Src A enhanced the activity approximately 5 to 10 fold over that seen with untreated samples.

Aliquots of the above whole tissue lysates were also measured for endogenous ERK phosphorylation by immunoblotting with an anti-phospho-ERK antibody as shown in Figure 6B. For this assessment, 10 day old CAMs were infected with either mock RCAS or RCAS that expresses SRC A. After two days, CAMs were dissected, cryopreserved in OCT and sectioned at 4 μm. Sections were immunostained with an anti-phosphorylated ERK antibody (New England Biolabs), washed and detected with a goat anti-rabbit

FITC-conjugated secondary antibody. Fluorescent images were captured on a cooled-CCD camera (Princeton Inst.). The photomicrographs indicate enhanced immunofluorescence with Src A-treated preparations compared to mock controls.

2. The Effect of Intradermal Expression of VEGF In $Src^{-/-}$ or $Src^{+/-}$ Mice Ears

Continuing the results obtained with chicken and mouse models, a direct genetic approach was employed to examine intradermal VEGF-induced angiogenesis in $Src^{-/-}$ mice. Also examined were effects on vascular permeability, since it was known that VEGF both initiates new blood vessel growth and can promote vascular permeability (Senger et al., 1983 Science 218:983-985; Ferrera and Davis-Smyth, 1997, Endocr.Rev. 16:4-25).

Intradermal injections of adenovirus expressing a human VEGF cDNA were performed in the ear of $Src^{-/-}$ and $Src^{+/-}$ mice, while control β-galactosidase expressing adenovirus was injected into the opposite ear of each mouse. VEGF-dependent new blood vessel growth in $Src^{+/-}$ ears was first detectable within 48 hr, and neovascularization was analyzed after 5 days.

Briefly, $pp60^{c\text{-}Src}$, $pp62^{c\text{-}yes}$, $pp59^{c\text{-}fyn}$, deficient mice (129/8v/Ev x C57B16/J) were generated as previously described (Soriano et al., 1991, Cell 64:693-702). Additional stocks were obtained from Jackson labs. Mouse ears were injected intradermally (Eriksson et al., 1980, Microvasc.Res. 19:374-378) with 5 μl of adenovirus expressing either VEGF or β-galactosidase and the ears photographed after 5 days, with a stereoscope.

It was found that there were identical viral expression

levels in $Src^{+/-}$ and $Src^{-/-}$ as determined by X-gal staining of β-galactosidase-adenovirus injected ears. In VEGF-injected $Src^{-/-}$ ears, there was no significant decrease in angiogenesis as measured by counting branch points ($p<0.05$). However, surprisingly, the most apparent phenotype in these animals was the complete blockade of vascular leakage compared to the VEGF-injected $Src^{+/-}$ ears. Examination of ears injected with VEGF confirms the extent of the vascular leakage in $Src^{+/-}$ mice, that is essentially absent in the $Src^{-/-}$ mice. The vascular leakage in these animals suggested that the VP activity, which has been associated with angiogenesis in vivo (Dvorak et al., 1995, Am.J.Pathol. 148:1029-1039), could be selectively disrupted in $pp60^{c\text{-}Src}$ deficient mice.

3. VEGF Fails to Compromise the Blood-Brain Barrier in Mice Lacking $pp60^{c\text{-}Src}$

The brain vasculature is characterized by a highly restrictive blood-brain barrier that prohibits small molecules from extravasating into the surrounding brain tissue. Tumor growth within the brain can compromise this barrier due in part to the production of angiogenic growth factors such as VEGF. Therefore, we examined the nature of the blood-brain barrier in $Src^{+/-}$ or $Src^{-/-}$ mice. In this case, VEGF or saline was stereotactically injected into the right or left hemisphere of the brain, respectively. All mice received systemic injections of Evan's blue dye to monitor VP activity.

Briefly, Saline or VEGF (200 ng in 2 ul) was injected stereotactically into the left or right frontal lobe 92 mm to the left/right of the midline, 0.5 mm rostral from bregma, and 3 mm in depth from the dura, respectively. The animals received

an Evan's blue dye solution intravenously 30 min after injection, as described above. After an additional 30 min, the mice were perfused and the brains were removed. Evan's blue dye fluorescence was observed using confocal laser microscopy of fresh unfixed cryosections of the brain.

Vascular leakage of blood was localized to the VGEF-injected hemisphere in $Src^{+/-}$ mice, but there was a complete absence of vascular leakage in $Src^{-/-}$ mice. This was also the case when examing the VP by measuring the accumulation of Evan's blue dye as detected by epifluoresence analysis of cryostat sections of these brains. Thus, VEGF compromises the blood-brain barrier in a manner that depends on active $pp60^{c\text{-}Src}$.

4. VEGF-Mediated VP, but Not Inflammation-Associated VP, Depends on $pp60^{c\text{-}Src}$

To further analyze and quantitate the effect of VEGF as a VP factor in $Src^{+/-}$ or $Src^{-/-}$ mice, a Miles assay (Miles & Miles, 1952) was used to quantitatively measure the vascular permeability in the skin of these animals. VEGF was injected intradermally in $Src^{+/-}$ or $Src^{-/-}$ mice that had received an intravenous systemic administration of Evan's blue dye. Within 15 min after injection of VEGF, there was a 3-fold increase in VP in $Src^{+/-}$ mice. However, in $Src^{-/-}$ mice no detectable VP activity was observed. Dye elution of the injected skin patches were quantitated and compared with control saline and bFGF. bFGF or saline controls injected adjacent to the VEGF showed no significant increase in VP.

Briefly, the Miles assay (Miles et al., 1952) was adapted for mice by injecting 10 μl of VEGF (400 ng/ml), allyl isothiocyanate (mustard oil, 20% w/v in mineral oil), or saline

intradermally into mice that had previously been intravenously injected with 100 μl of 0.5% Evan's blue dye. After 15 min, the skin patches were dissected, photographed, and eluted at 58°C with formalin and quantitated with a spectrophotometer.

Vascular leakage/permeability is also known to occur during inflammation, which allows for the accumulation of serum-associated adhesive protein and inflammatory cells in tissues. In fact, inflammatory mediators themselves directly promote vascular leakage. Therefore, one such inflammatory mediator, allyl isothiocyanate, also known as mustard oil (Inoue et al., 1997, supra), was tested in $Src^{+/-}$ or $Src^{-/-}$ mice for its capacity to produce VP. Unlike that observed in VEGF-stimulated $Src^{-/-}$ animals, no decrease in the VP produced by the injection of the inflammatory mediator allyl isothiocyanate was detected. Thus, it can be concluded that Src plays a selective role in the VP activity induced with VEGF and does not influence VP associated with the inflammatory process.

5. VEGF-Mediated VP changes Depends on activity of Src and Yes, but not Fyn

The specificity of the Src requirement for VP was explored by examining the VEGF-induced VP activity associated with SFKs such as Fyn or Yes, which, like Src, are known to be expressed in endothelial cells (Bull et al., 1994, FEBS Letters, 361:41-44; Kiefer et al., 1994, Curr.Biol. 4:100-109). It was confirmed that these three SFKs were expressed equivalently in the aortas of wild-type mice. Like $Src^{-/-}$ mice, animals deficient in Yes were also defective in VEGF-induced VP. However, surprisingly, mice lacking Fyn retained a high VP in response to VEGF that was not significantly different from control animals. The disruption of

VEGF-induced VP in $Src^{-/-}$ or $yes^{-/-}$ mice demonstrates that the kinase activity of specific SFKs is essential for VEGF-mediated signalling event leading to VP activity but not angiogenesis.

The vascular permeability properties of VEGF in the skin of $Src^{+/-}$ (Figure 11A, left panel) or $Src^{-/-}$ (Figure 11A, right panel) mice was determined by intradermal injection of saline or VEGF (400 ng) into mice that have been intravenously injected with Evan's blue dye. After 15 min, skin patches were photographed (scale bar, 1 mm). The stars indicate the injection sites. The regions surrounding the injection sites of VEGF, bFGF or saline were dissected, and the VP quantitatited by elution of the Evan's blue dye in formamide at 58°C for 24 hr, and the absorbance measured at 500 nm (Figure 11B, left graph). The ability of an inflammation mediator (allyl isothiocyanate), known to induce inflammation related VP, was tested in $Src^{+/-}$ or $Src^{-/-}$ mice (Figure 11B, right).

The ability of VEGF to induce VP was compared in $Src^{-/-}$, $fyn^{-/-}$, or $yes^{-/-}$ mice in the Miles assay (Figure 11C).Data for each of the Miles assays are expressed as the mean ± SD of triplicate animals. $Src^{-/-}$ and $yes^{-/-}$ VP defects compared to control animals were statistically significant (*$p < 0.05$, paired t test), whereas the VP defects in neither the VEGF-treated $fyn^{-/-}$ mice nor the allyl isothiocyanate treated $Src^{+/-}$ mice were statistically significant (**$p<0.05$).

6. <u>Src family tyrosine kinase inhibitor treated mice, and Src -/- mice show reduced tissue damage associated with trauma or injury to blood vessels than untreated wild-type mice</u>

Specific administration of inhibitors of the Src family kinases acts as inhibitors of pathological vascular leakage and

permeability during vascular injury or disorders such as stroke. The vascular endothelium is a dynamic cell type that responds to many cues to regulate processes such as the sprouting of new blood vessels during angiogenesis of a tumor, to the regulation of the permeability of the vessel wall during stroke--induced edema and tissue damage.

Reduction of vascular permeability in two mouse stroke models, by drug inhibition of the Src pathway, is sufficient to inhibit brain damage by reducing ischemia-induced vascular leak. Furthermore, in mice genetically deficient in Src, which have reduced vascular leakage/permeability, infarct volume is also reduced. The combination of the synthetic Src inhibitor data, with the supporting genetic evidence of reduced the vascular leakage in stroke and other related models demonstrates the physiological relevance of this approach in reducing brain damage following strokes. Inhibition of these pathways with a range of available Src family kinase inhibitors of these signaling cascades has the therapeutic benefit of mitigating brain damage from vascular permeability-related tissue damage.

Two different methods for induction of focal cerebral ischemia were used. Both animal models of focal cerebral ischemia are well established and widely used in stroke research. Both models have been previously used to investigate the pathophysiology of cerebral ischemia as well as to test novel antistroke drugs.

a) Mice were anesthetized with avertin and body temperature was maintained by keeping the animal on a heating pad. A incision was made between the right ear and the right eye. The scull was exposed by retraction of the temporal muscle and a small burr hole was drilled in the region over the middle

cerebral artery (MCA). The meninges were removed and the right MCA was occluded by coagulation using a heating filament. The animals were allowed to recover and were returned to their cages. After 24 hours, the brains were perfused, removed and cut into 1 mm cross-sections. The sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) and the infarcted brain area was identified as unstained (white) tissue surrounded by viable (red) tissue. The infarct volume was defined as the sum of the unstained areas of the sections multiplied by their thickness.

Mice deficient in Src (Src-/-) were used to study the role of Src in cerebral ischemia. Src+/- mice served as controls. We found that in Src-/- mice the infarct volume was reduced from 26 ± 10 mm^3 to 16 ± 4 mm^3 in controls 24 hours after the insult. The effect was even more pronounced when C57Bl6 wild-type mice were injected with 1.5 mg/kg PP1 intraperitoneally (i.p.) 30 min after the vessel occlusion. The infarct size was reduced from 31 ± 12 mm^3 in the untreated group to 8 ± 2 mm^3 in the PP1-treated group.

b) In a second model of focal cerebral ischemia the MCA was occluded by placement of an embolus at the origin of the MCA. A single intact fibrin-rich 24 h old homologous clot was placed at the origin of the MCA using a modified PE-50 catheter. Induction of cerebral ischemia was proven by the reduction of cerebral blood flow in the ipsilateral hemisphere compared to the contralateral hemisphere. After 24 hours the brains were removed, serial sections were prepared and stained with hematoxylin-eosin (HE). Infarct volumes were determined by adding the infarct areas in serial HE sections multiplied by the distance between each section.

The dosage of PP1 used in this study (1.5 mg/kg i.p.) was empirically chosen. It is known that VEGF is first expressed about 3 hours after cerebral ischemia in the brain with a maximum after 12 to 24 hours. In this study PP1 was given 30 min after the onset of the infarct to completely block VEGF-induced vascular permeability increase. According to the time course of typical VEGF expression, a potential therapeutical window for the administration of Src-inhibitors would be up to 12 hours after the stroke. In diseases associated with a sustained increase in vascular permeability a chronic administration of the Src inhibiting drug is appropriate.

Figure 12 is a graph which depicts the comparative results of averaged infarct volume (mm^3) in mouse brains after injury, where mice were heterogeneous Src (Src +/-), dominant negative Src mutants (Src -/-), wild type mice (WT), or wild type mice treated with 1.5 mg/kg PP1 (PP1).

Figure 13 illustrates sample sequential MRI scans of isolated perfused mouse brain after treatment to induce CNS injury, where the progression of scans in the PP1 treated animal (right) clearly shows less infarct than the progression of scans in the control untreated animal (left).

The methods of the present invention are particularly suited for the specific intervention of VP induced tissue damage because the targeted inhibition of Src family tyrosine kinase action focuses inhibition on VP without long term effect on other VEGF-induced responses which can be beneficial to recovery from injury. In contrast to neutralizing VEGF protein, the inhibition of Src does not interfere with the cumulative angiogenic effect of VEGF which might be beneficial in a later stage of the disease.

The use of synthetic small-molecule inhibitors is in general safer and more manageable that the use of large proteins. The use of recombinant proteins, such as a VEGF receptor-murine Immunoglobulin fusion protein is potentially harmful, and does not allow for repeated administration for fear of provoking an allergic reaction when used in humans (i.e. Human anti-mouse antibody; HAMA).

Finally, VEGF is not the only activator of downstream Src, other cytokines involved in the pathophysiology of cerebral ischemia which can influence vascular permeability, such as IL-6 and TNF-α. Thus, inhibition of VEGF may not inhibit all subsequent injury related Src activation. In fact, reduction of infarct size by PP1 is more pronounced than by VEGF antagonism indicating that other pathways may activate Src kinases facilitating permeability increase.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

We Claim:

1. A method for ameliorating tissue damage related to vascular leakage or edema comprising contacting said tissue with a vascular permeability modulating amount of a pharmaceutical composition comprising a Src family tyrosine kinase inhibitor.

2. The method of claim 1 wherein said Src family tyrosine kinase inhibitor is a chemical inhibitor.

3. The method of claim 2 wherein said chemical inhibitor is selected from the group consisting of PP1, PP2, PD173955, AGL1872, PD162531, Radicicol R2146, and Geldanamycin.

4. The method of claim 3 wherein said inhibitor is PP1.

5. The method of claim 1 wherein said Src family tyrosine kinase inhibitor is an inactive Src protein.

6. The method of claim 5 wherein said inactive Src protein is Src K295M.

7. The method of claim 5 wherein said inactive Src protein is Src 251.

8. The method of claim 1 wherein said Src family tyrosine kinase inhibitor is an inactive Yes protein.

9. The method of claim 1 wherein said Src family tyrosine kinase inhibitor is active c-terminal Src Kinase (CSK) protein.

10. A method of claim 1 wherein said Src family tyrosine kinase inhibitor is a nucleic acid encoding for a Src family tyrosine kinase inhibitor protein.

11. The method of claim 10 wherein said pharmaceutical composition includes a retroviral expression vector.

12. The method of claim 10 wherein said pharmaceutical composition includes a non-viral expression vector.

13. A method of claim 10 wherein said inhibitor protein is selected from the group consisting of inactive Src protein, inactive Yes protein, active c-terminal Src kinase (CSK), and a mixture thereof.

14. The method of claim 13 wherein said inactive Src protein is Src K295M.

15. The method of claim 13 wherein said inactive Src protein is Src 251.

16. A method of claim 1 wherein said inhibitor is a Src tyrosine kinase inhibitor.

17. An article of manufacture comprising packaging material and a pharmaceutical composition contained within said packaging material, wherein said pharmaceutical composition is capable of modulating vascular permeability increase in a tissue suffering from a disease condition, wherein said packaging material comprises a label which indicates that said pharmaceutical composition can be used for treatment of vascular leakage or edema associated disease conditions, and wherein said pharmaceutical composition comprises a Src family tyrosine kinase inhibitor and a pharmaceutically acceptable carrier therefor.

18. An article of manufacture of claim 17 wherein said Src family tyrosine kinase inhibitor is a chemical inhibitor.

19. An article of manufacture of claim 18 wherein said Src family tyrosine kinase inhibitor is selected from the group consisting of PP1, PP2, PD173955, AGL1872, PD162531, Radicicol R2146, and Geldanamycin.

20. An article of manufacture of claim 18 wherein said Src family tyrosine kinase inhibitor is PP1.

21. An article of manufacture of claim 17 wherein said Src family tyrosine kinase inhibitor is inactive Src protein.

22. An article of manufacture of claim 21 wherein said inactive Src protein is Src K295M.

23. An article of manufacture of claim 21 wherein said inactive Src protein is Src 251.

24. An article of manufacture of claim 17 wherein said Src family tyrosine kinase inhibitor is inactive Yes protein.

25. An article of manufacture of claim 17 wherein said Src family tyrosine kinase inhibitor is active c-terminal Src Kinase (CSK) protein.

26. An article of manufacture comprising packaging material and a pharmaceutical composition contained within said packaging material, wherein said pharmaceutical composition is capable of modulating vascular permeability in a tissue suffering from a disease condition, wherein said packaging material comprises a label which indicates that said pharmaceutical composition can be used for treatment of vascular leakage or edema associated disease conditions, and wherein said pharmaceutical composition comprises nucleic acid encoding for a Src family tyrosine kinase inhibitor, in a pharmaceutically acceptable carrier.

27. An article of manufacture of claim 26 wherein said Src family tyrosine kinase inhibitor is inactive Src protein.

28. An article of manufacture of claim 27 wherein said inactive Src protein is Src K295M.

29. An article of manufacture of claim 27 wherein said inactive Src protein is Src 251.

30. An article of manufacture of claim 26 wherein said Src family tyrosine kinase inhibitor is inactive Yes protein.

31. An article of manufacture of claim 26 wherein said Src family tyrosine kinase inhibitor is active c-terminal Src Kinase (CSK) protein.

ABSTRACT

The present invention describes methods for ameliorating tissue damage due to vascular leakage or edema by modulating vascular permeability (VP) in tissues using an inhibitor of Src family tyrosine kinases. Related compositions and articles of manufacture are also disclosed.

CHICKEN c-SRC cDNA

(SEQ ID NO:2)

1 tctgacaccc atctgtctgt ctgtctgtgt gctgcaggag ctgagctgac tctgctgtgg
61 cctcgcgtac cactgtggcc aggcggtagc tgggacgtgc agcccaccac catggggagc
121 agcaagagca agcccaagga ccccagccag cgccggcgca gcctggagcc acccgacagc
181 acccaccacg ggggattccc agcctcgcag acccccaaca agacagcagc ccccgacacg
241 caccgcaccc ccagccgctc ctttgggacc gtggccaccg agcccaagct cttcgggggc
301 ttcaacactt ctgacaccgt tacgtcgccg cagcgtgccg gggcactggc tggcggcgtc
361 accactttcg tggctctcta cgactacgag tcccggactg aaacggactt gtccttcaag
421 aaaggagaac gcctgcagat tgtcaacaac acggaaggtg actggtggct ggctcattcc
481 ctcactacag gacagacggg ctacatcccc agtaactatg tcgcgcccte agactccatc
541 caggctgaag agtggtactt tgggaagatc actcgtcggg agtccgagcg gctgctgctc
601 aaccccgaaa acccccgggg aaccttcttg gtccgggaga gcgagacgac aaaaggtgcc
661 tattgcctct ccgtttctga ctttgacaac gccaaggggc tcaatgtgaa gcactacaag
721 atccgcaagc tggacagcgg cggcttctac atcacctcac gcacacagtt cagcagcctg
781 cagcagctgg tggcctacta ctccaaacat gctgatggct tgtgccaccg cctgaccaac
841 gtctgcccca cgtccaagcc ccagacccag ggactcgcca aggacgcgtg ggaaatcccc
901 cgggagtcgc tgcggctgga ggtgaagctg gggcagggct gctttggaga ggtctggatg
961 gggacctgga acggcaccac cagagtggcc ataaagactc tgaagcccgg caccatgtcc
1021 ccggaggcct tcctgcagga agcccaagtg atgaagaagc tccggcatga gaagctggtt
1081 cagctgtacg cagtggtgtc ggaagagccc atctacatcg tcactgagta catgagcaag
1141 gggagcctcc tggatttcct gaagggagag atgggcaagt acctgcggct gccacagctc
1201 gtcgatatgg ctgctcagat tgcatccggc atggcctatg tggagaggat gaactacgtg
1261 caccgagacc tgcgggcggc caacatcctg gtgggggaga acctggtgtg caaggtggct
1321 gactttgggc tggcacgcct catcgaggac aacgagtaca cagcacggca aggtgccaag
1381 ttccccatca agtggacagc ccccgaggca gccctctatg gccggttcac catcaagtcg
1441 gatgtctggt ccttcggcat cctgctgact gagctgacca ccaagggccg ggtgccatac
1501 ccagggatgg tcaacaggga ggtgctggac caggtggaga ggggctaccg catgccctgc
1561 ccgcccgagt gccccgagtc gctgcatgac ctcatgtgcc agtgctggcg gagggaccct
1621 gaggagcggc ccacttttga gtacctgcag gccttcctgg aggactactt cacctcgaca
1681 gagccccagt accagcctgg agagaaccta taggcctgga gctcctcctg gaccagaggc
1741 ctcgctgtgg ggtacaggg

FIG. 1

CHICKEN cSRC ENCODED PROTEIN

(SEQ ID NO:3)

MGSSKSKPKDPSQRRRSLEPPDSTHHGGFPASQTPNKTAA

PDTHRTPSRSFGTVATEPKLFGGFNTSDTVTSPQRAGALA

GGVTTFVALYDYESRTETDLSFKKGERLQIVNNTEGDWWL

AHSLTTGQTGYIPSNYVAPSDSIQAEEWYFGKITRRESER

LLLNPENPRGTFLVRESETTKGAYCLSVSDFDNAKGLNVK

HYKIRKLDSGGFYITSRTQFSSLQQLVAYYSKHADGLCHR

LTNVCPTSKPQTQGLAKDAWEIPRESLRLEVKLGQGCFGE

VWMGTWNGTTRVAIKTLKPGTMSPEAFLQEAQVMKKLRHE

KLVQLYAVVSEEPIYIVTEYMSKGSLLDFLKGEMGKYLRL

PQLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENL

VCKVADFGLARLIEDNEYTARQGAKFPIKWTAPEAALYGR

FTIKSDVWSFGILLTELTTKGRVPYPGMVNREVLDQVERG

YRMPCPPECPESLHDLMCQCWRRDPEERPTFEYLQAFLE

DYFTSTEPQYQPGENL

FIG. 2

HUMAN c-SRC cDNA

(SEQ ID NO:4)

1 gcgccgcgtc ccgcaggccg tgatgccgcc cgcgcggagg tggcccggac cgcagtgccc
61 caagagagct ctaatggtac caagtgacag gttggcttta ctgtgactcg gggacgccag
121 agctcctgag aagatgtcag caatacaggc cgcctggcca tccggtacag aatgtattgc
181 caagtacaac ttccacggca ctgccgagca ggacctgccc ttctgcaaag gagacgtgct
241 caccattgtg gccgtcacca aggaccccaa ctggtacaaa gccaaaaaca aggtgggccg
301 tgagggcatc atcccagcca actacgtcca gaagcgggag ggcgtgaagg cgggtaccaa
361 actcagcctc atgccttggt tccacggcaa gatcacacgg gagcaggctg agcggcttct
421 gtacccgccg gagacaggcc tgttcctggt gcgggagagc accaactacc ccggagacta
481 cacgctgtgc gtgagctgcg acggcaaggt ggagcactac cgcatcatgt accatgccag
541 caagctcagc atcgacgagg aggtgtactt tgagaacctc atgcagctgg tggagcacta
601 cacctcagac gcagatggac tctgtacgcg cctcattaaa ccaaaggtca tggagggcac
661 agtggcggcc caggatgagt tctaccgcag cggctgggcc ctgaacatga aggagctgaa
721 gctgctgcag accatcggga agggggagtt cggagacgtg atgctgggcg attaccgagg
781 gaacaaagtc gccgtcaagt gcattaagaa cgacgccact gcccaggcct tcctggctga
841 agcctcagtc atgacgcaac tgcggcatag caacctggtg cagctcctgg gcgtgatcgt
901 ggaggagaag ggcgggctct acatcgtcac tgagtacatg gccaagggga gccttgtgga
961 ctacctgcgg tctaggggtc ggtcagtgct gggcggagac tgtctcctca agttctcgct
1021 agatgtctgc gaggccatgg aatacctgga gggcaacaat ttcgtgcatc gagacctggc
1081 tgcccgcaat gtgctggtgt ctgaggacaa cgtggccaag gtcagcgact ttggtctcac
1141 caaggaggcg tccagcaccc aggacacggg caagctgcca gtcaagtgga cagcccctga
1201 ggccctgaga gagaagaaat tctccactaa gtctgacgtg tggagtttcg gaatccttct
1261 ctgggaaatc tactcctttg ggcgagtgcc ttatccaaga attcccctga aggacgtcgt
1321 ccctcgggtg gagaagggct acaagatgga tgcccccgac ggctgcccgc ccgcagtcta
1381 tgaagtcatg aagaactgct ggcacctgga cgccgccatg cggccctcct tcctacagct
1441 ccgagagcag cttgagcaca tcaaaaccca cgagctgcac ctgtgacggc tggcctccgc
1501 ctgggtcatg ggcctgtggg gactgaacct ggaagatcat ggacctggtg cccctgctca
1561 ctgggcccga gcctgaactg agccccagcg ggctggcggg ccttttcct gcgtcccagc
1621 ctgcaccct ccggcccgt ctctcttgga cccacctgtg gggcctgggg agcccactga
1681 ggggccaggg aggaaggagg ccacggagcg ggaggcagcg ccccaccacg tcgggcttcc
1741 ctggcctccc gccactcgcc ttcttagagt tttattcctt tccttttttg agatttttt
1801 tccgtgtgtt tatttttat tatttttcaa gataaggaga aagaaagtac ccagcaaatg
1861 ggcattttac aagaagtacg aatcttattt ttcctgtcct gcccgtgagg gtgggggga
1921 ccgggccccc ctctagggac ccctcgcccc agcctcattc cccattctgt gtcccatgtc
1981 ccgtgtctcc tcggtcgccc cgtgtttgcg cttgaccatg ttgcactgtt tgcatgcgcc
2041 cgaggcagac gtctgtcagg ggcttggatt tcgtgtgccg ctgccacccg cccacccgcc
2101 ttgtgagatg gaattgtaat aaaccacgcc atgaggacac cgccgcccgc ctcggcgctt
2161 cctccaccga aaaaaaaaaa aaaaaaa

FIG. 3

HUMAN c-SRC ENCODED PROTEIN

(SEQ ID NO:5)

MSAIQAAWPSGTECIAKYNFHGTAEQDLPFCKGDVLTIVAVTKD

PNWYKAKNKVGREGIIPANYVQKREGVKAGTKLSLMPWFHGKIT

REQAERLLYPPETGLFLVRESTNYPGDYTLCVSCDGKVEHYRIMY

HASKLSIDEEVYFENLMQLVEHYTSDADGLCTRLIKPKVMEGTVA

AQDEFYRSGWALNMKELKLLQTIGKGEFGDVMLGDYRGNKVAV

KCIKNDATAQAFLAEASVMTQLRHSNLVQLLGVIVEEKGGLYIVTE

YMAKGSLVDYLRSRGRSVLGGDCLLKFSLDVCEAMEYLEGNNFVH

RDLAARNVLVSEDNVAKVSDFGLTKEASSTQDTGKLPVKWTAPEAL

REKKFSTKSDVWSFGILLWEIYSFGRVPYPRIPLKDVVPRVEKGYKM

DAPDGCPPAVYEVMKNCWHLDAAMRPSFLQLREQLEHIKTHELHL

FIG. 4

Activation of endogenous Src activity by bFGF and VEGF

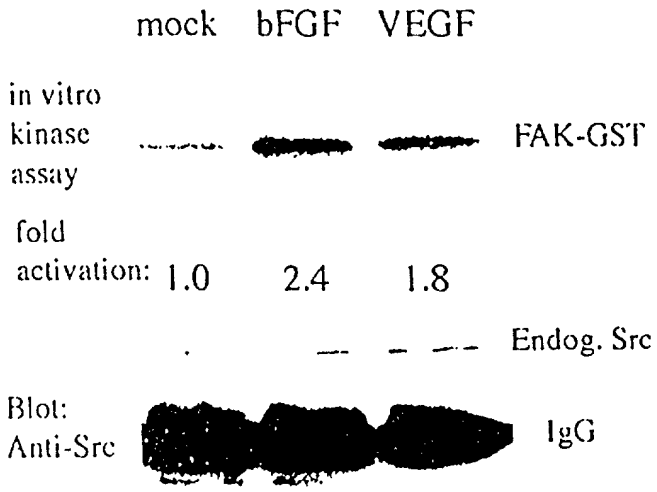
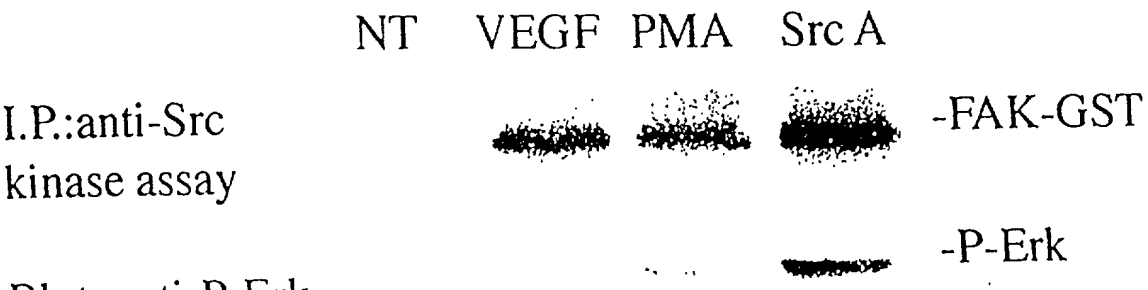


FIG. 5

Retroviral expression of Src A activates vascular MAP kinase phosphorylation



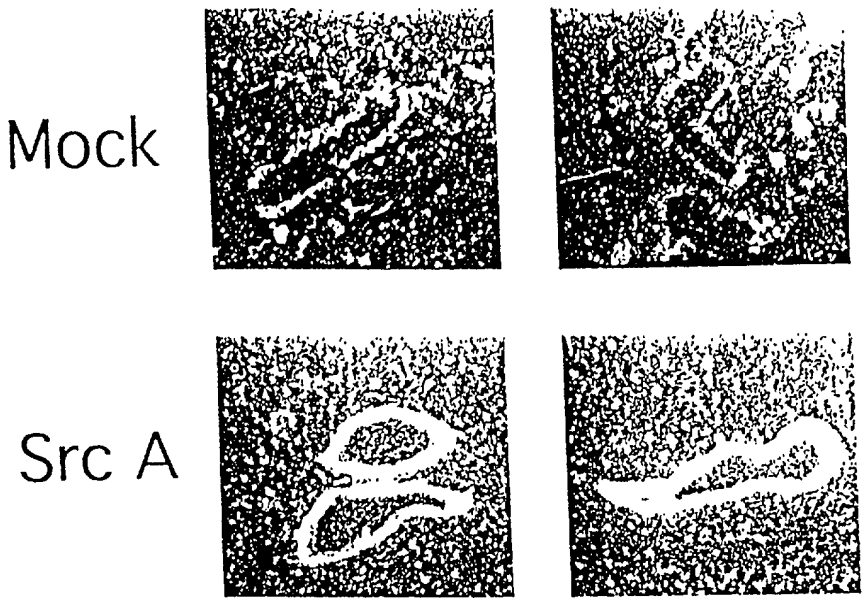


FIG. 6

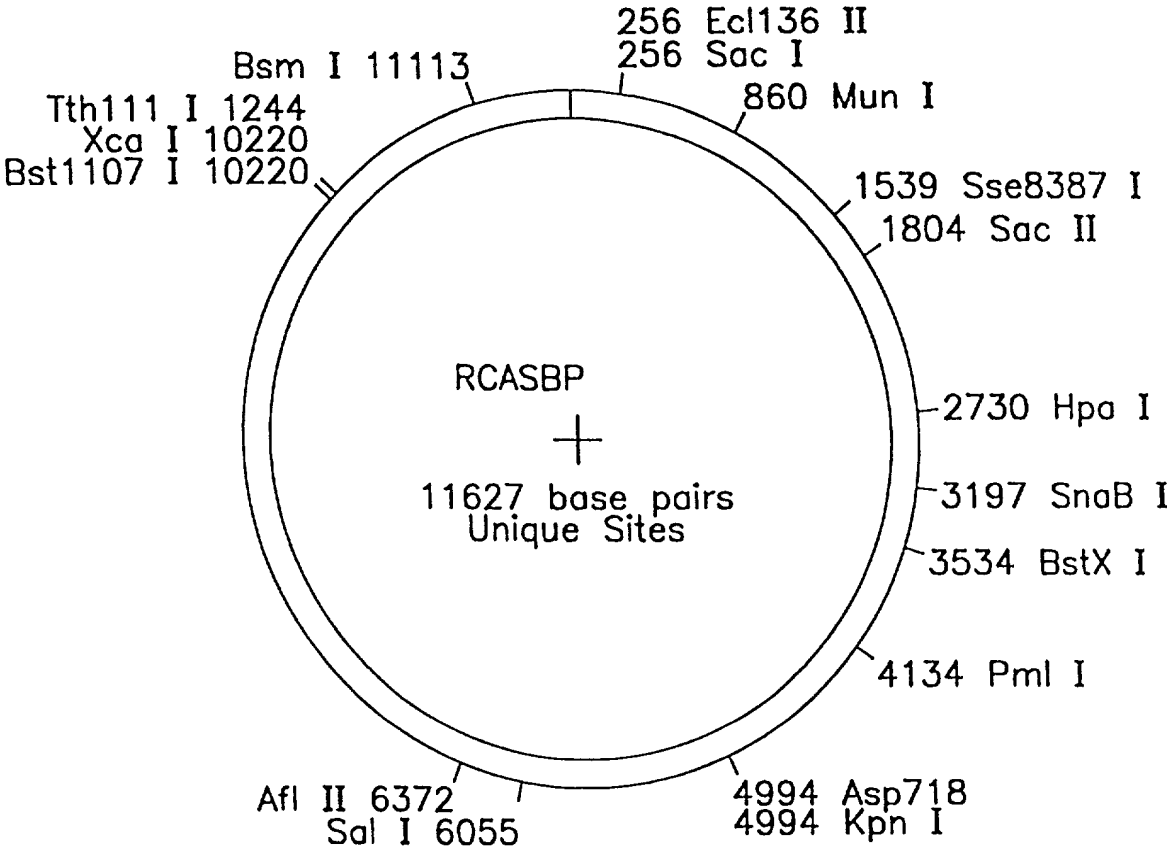

256 Ecl136 II
256 Sac I
860 Mun I
1539 Sse8387 I
1804 Sac II
2730 Hpa I
3197 SnaB I
3534 BstX I
4134 Pml I
4994 Asp718
4994 Kpn I
Afl II 6372
Sal I 6055
Bsm I 11113
Tth111 I 1244
Xca I 10220
Bst1107 I 10220
RCASBP
11627 base pairs
Unique Sites

FIG. 7

human Yes-1 Protein amino acid sequence

"MGCIKSKENKSPAIKYRPENTPEPVSTSVSHYGAEPTTVSPCPS
SSAKGTAVNFSSLSMTPFGGSSGVTPFGGASSSFSVVPSSYPAGLTGGVTIFVALYDY
EARTTEDLSFKKGERFQIINNTEGDWWEARSIATGKNGYIPSNYVAPADSIQAEEWYF
GKMGRKDAERLLLNPGNQRGIFLVRESETTKGAYSLSIRDWDEIRGDNVKHYKIRKLD
NGGYYITTRAQFDTLQKLVKHYTEHADGLCHKLTTVCPTVKPQTQGLAKDAWEIPRES
LRLEVKLGQGCFGEVWMGTWNGTTKVAIKTLKPGTMMPEAFLQEAQIMKKLRHDKLVP
LYAVVSEEPIYIVTEFMSKGSLLDFLKEGDGKYLKLPQLVDMAAQIADGMAYIERMNY
IHRDLRAANILVGENLVCKIADFGLARLIEDNEYTARQGAKFPIKWTAPEAALYGRFT
IKSDVWSFGILQTELVTKGRVPYPGMVNREVLEQVERGYRMPCPQGCPESLHELMNLC
WKKDPDERPTFEYIQSFLEDYFTATEPQYQPGENL"

FIGURE 8

FIGURE 9

```
   1 gcggagccaa ggcacacggg tctgaccctt gggccggccc ggagcaagtg acacggaccg
  61 gtcgcctatc ctgaccacag caaagcggcc cggagcccgc ggaggggacc tgacgggggc
 121 gtaggcgccg gaaggctggg ggccccggag ccgggccggc gtggcccgag ttccggtgag
 181 cggacggcgg cgcgcgcaga tttgataatg ggctgcatta aaagtaaaga aaacaaaagt
 241 ccagccatta aatacagacc tgaaaatact ccagagcctg tcagtacaag tgtgagccat
 301 tatggagcag aacccactac agtgtcacca tgtccgtcat cttcagcaaa gggaacagca
 361 gttaatttca gcagtctttc catgacacca tttggaggat cctcaggggt aacgcctttt
 421 ggaggtgcat cttcctcatt ttcagtggtg ccaagttcat atcctgctgg tttaacaggt
 481 ggtgttacta tatttgtggc cttatatgat tatgaagcta gaactacaga agacctttca
 541 tttaagaagg gtgaaagatt tcaaataatt aacaatacgg aaggagattg gtgggaagca
 601 agatcaatcg ctacaggaaa gaatggttat atcccgagca attatgtagc gcctgcagat
 661 tccattcagg cagaagaatg gtattttggc aaaatgggga gaaaagatgc tgaaagatta
 721 cttttgaatc ctggaaatca acgaggtatt ttcttagtaa gagagagtga aacaactaaa
 781 ggtgcttatt ccctttctat tcgtgattgg gatgagataa ggggtgacaa tgtgaaacac
 841 tacaaaatta ggaaacttga caatggtgga tactatatca caaccagagc acaatttgat
 901 actctgcaga aattggtgaa acactacaca gaacatgctg atggtttatg ccacaagttg
 961 acaactgtgt gtccaactgt gaaacctcag actcaaggtc tagcaaaaga tgcttgggaa
1021 atccctcgag aatctttgcg actagaggtt aaactaggac aaggatgttt cggcgaagtg
1081 tggatgggaa catggaatgg aaccacgaaa gtagcaatca aaacactaaa accaggtaca
1141 atgatgccag aagctttcct tcaagaagct cagataatga aaaaattaag acatgataaa
1201 cttgttccac tatatgctgt tgtttctgaa gaaccaattt acattgtcac tgaatttatg
1261 tcaaaaggaa gcttattaga tttccttaag gaaggagatg gaaagtattt gaagcttcca
1321 cagctggttg atatggctgc tcagattgct gatggtatgg catatattga aagaatgaac
1381 tatattcacc gagatcttcg ggctgctaat attcttgtag gagaaaatct tgtgtgcaaa
1441 atagcagact ttggtttagc aaggttaatt gaagacaatg aatacacagc aagacaaggt
1501 gcaaaatttc caatcaaatg gacagctcct gaagctgcac tgtatggtcg gtttacaata
1561 aagtctgatg tctggtcatt tggaattctg caaacagaac tagtaacaaa gggccgagtg
1621 ccatatccag gtatggtgaa ccgtgaagta ctagaacaag tggagcgagg atacaggatg
1681 ccgtgccctc agggctgtcc agaatccctc catgaattga tgaatctgtg ttggaagaag
1741 gaccctgatg aaagaccaac atttgaatat attcagtcct tcttggaaga ctacttcact
1801 gctacagagc cacagtacca gccaggagaa aatttataat tcaagtagcc tatttttatat
1861 gcacaaatct gccaaaatat aaagaacttg tgtagatttt ctacaggaat caaaagaaga
1921 aaatcttctt tactctgcat gtttttaatg gtaaactgga atcccagata tggttgcaca
1981 aaaccacttt ttttcccca agtattaaac tctaatgtac caatgatgaa tttatcagcg
2041 tatttcaggg tccaaacaaa atagagctaa gatactgatg acagtgtggg tgacagcatg
2101 gtaatgaagg acagtgaggc tcctgcttat ttataaatca tttccttttct ttttttcccc
2161 aaagtcagaa ttgctcaaag aaaattattt attgttacag ataaaacttg agagataaaa
2221 agctatacca taataaaatc taaaattaag gaatatcatg ggaccaaata attccattcc
2281 agtttttaa agtttcttgc atttattatt ctcaaaagtt ttttctaagt taaacagtca
2341 gtatgcaatc ttaatatatg ctttcttttg catggacatg ggccaggttt ttcaaaagga
2401 atataaacag gatctcaaac ttgattaaat gttagaccac agaagtggaa tttgaaagta
2461 taatgcagta cattaatatt catgttcatg gaactgaaag aataagaact ttttcacttc
2521 agtccttttc tgaagagttt gacttagaat aatgaaggta actagaaagt gagttaatct
2581 tgtatgaggt tgcattgatt ttttaaggca atatataatt gaaactactg tccaatcaaa
2641 ggggaaatgt tttgatcttt agatagcatg caaagtaaga cccagcattt taaaagccct
2701 tttttaaaaa ctagacttcg tactgtgagt attgcttata tgtccttatg gggatgggtg
2761 ccacaaatag aaaatatgac cagatcaggg acttgaatgc acttttgctc atggtgaata
2821 tagatgaaca gagaggaaaa tgtatttaaa agaaatacga gaaaagaaaa tgtgaaagtt
2881 ttacaagtta gagggatgga aggtaatgtt taatgttgat gtcatggagt gacagaatgg
2941 ctttgctggc actcagagct cctcacttag ctatattctg agactttgaa gagttataaa
3001 gtataactat aaaactaatt tttcttacac actaaatggg tatttgttca aaataatgaa
3061 gttatggctt cacattcatt gcagtgggat atggttttta tgtaaaacat ttttagaact
3121 ccagttttca aatcatgttt gaatctacat tcacttttt ttgttttctt ttttgagacg
3181 gagtctcgct ctgccgccca ggctggagtg cagtggcgcg atctcggctc actgcaagct
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3361 ttagccagga tggtctcgat ctcctgacct tgtgatctgc ccgcctcggc ctcccaaagt
3421 gctgggatta caggtgtgag ccaccgcgcc cagcctacat tcacttctaa agtctatgta
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3481 atggtggtca ttttttccct tttagaatac attaaatggt tgatttgggg aggaaaactt
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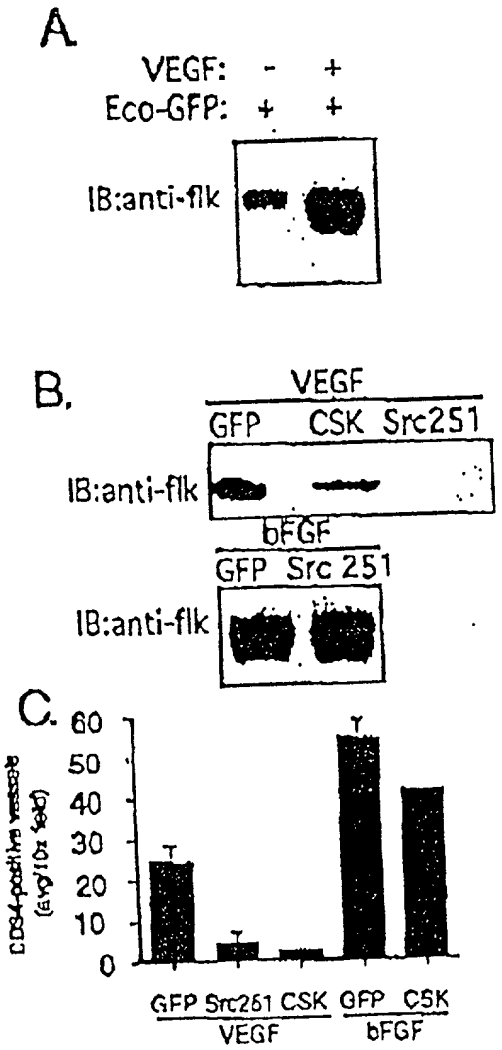
FIGURE 9 Con't


A
VEGF: - +
Eco-GFP: + +
IB:anti-flk
B.
VEGF
GFP CSK Src251
IB:anti-flk
bFGF
GFP Src 251
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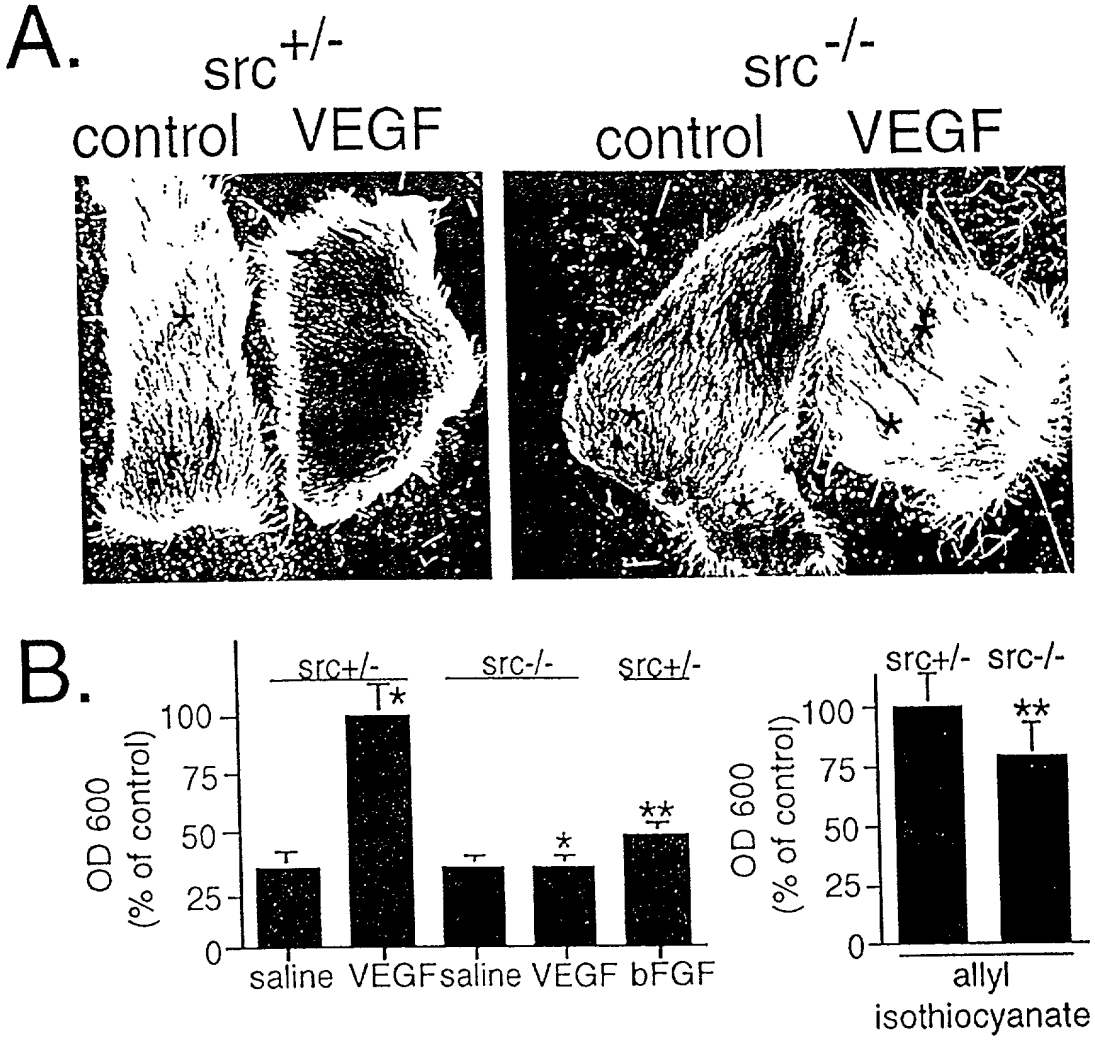
VEGF
bFGF

FIGURE 10


A.
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control
VEGF
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control
VEGF
B.
src+/-
src-/-
src+/-
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(% of control)
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saline
VEGF
saline
VEGF
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FIG. 11

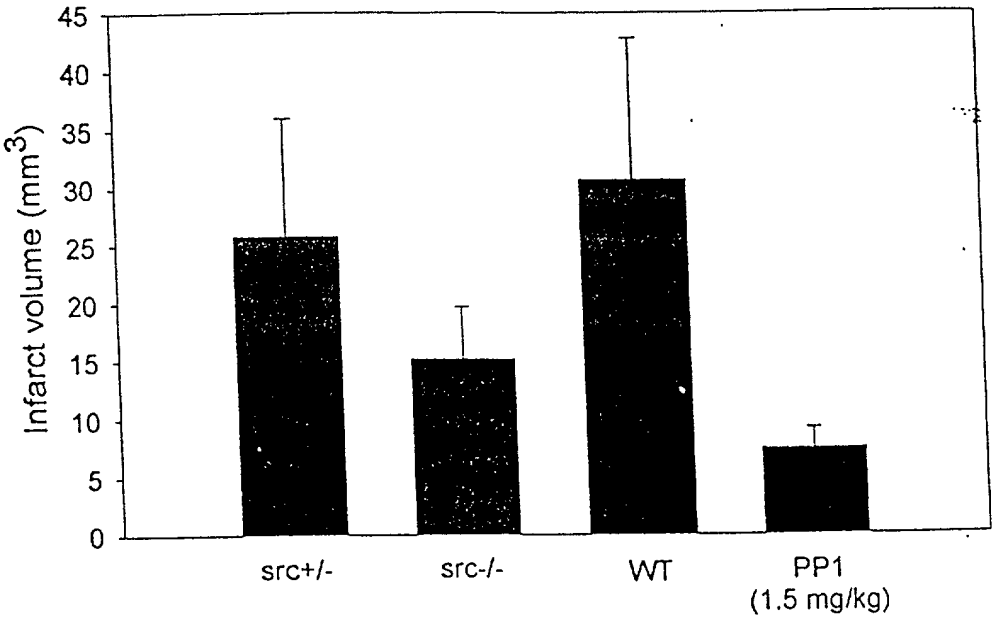
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FIG 12

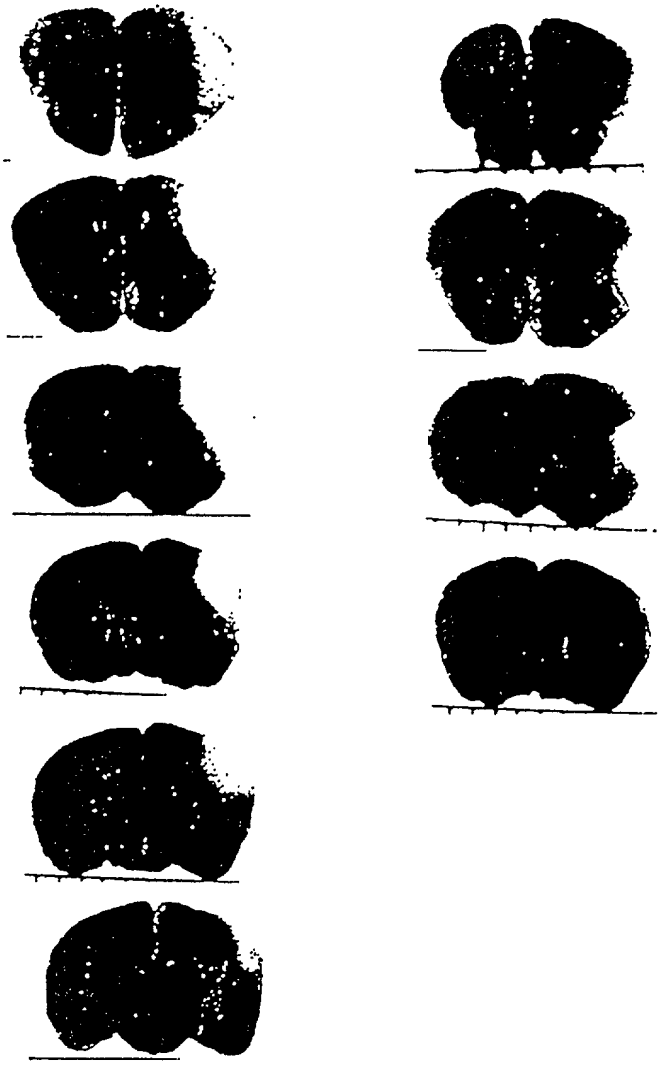

FIG 13

PATENT APPLICATION
DECLARATION AND POWER OF ATTORNEY

I HEREBY DECLARE THAT:

My residence, post office address, and citizenship are as stated next to my name in PART A on page 2 hereof.

I believe I am the original, first, and sole inventor (if only one name is listed) or an original, first, and joint inventor (if plural names are listed) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS USEFUL FOR TREATING VASCULAR LEAKAGE AND EDEMA SRC or YES TYROSINE KINASE INHIBITORS

the specification of which:

X is attached hereto;

__ was filed on _____ as Application Serial No. ____ and was amended on _____ (if applicable).

X was filed on May 28, 1999 as PCT International Application Number **PCT/US99/11780** and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to herein.

I acknowledge the duty to disclose information to the Patent and Trademark Office known to me to be material to the patentability of this application, as defined in Title 37, Code of Federal Regulations, Sec. 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Sec. 119 of any foreign application(s) for patent or inventor's certificate listed in PART B on page 2 hereof and have also identified in PART B on page 2 hereof any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

I hereby claim the benefit under Title 35, United States Code, Sec. 120 of any United States application(s) listed in PART C of page 2 hereof and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Sec. 112, I acknowledge the duty to disclose all information to the Patent and Trademark Office known to me to be material to patentability of this application, as defined in Title 37, Code of Federal Regulations, Sec. 1.56, which became available between the filing date of the prior application and the national or PCT international filing date of this application.

PATENT APPLICATION DECLARATION AND POWER OF ATTORNEY

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following as my attorneys or agents with full power of substitution to prosecute this application and transact all business in the United States Patent and Trademark Office connected therewith:

Michael A. Hierl	Reg. No. 29,807	Arne M. Olson	Reg. No. 30,203
Dolores T. Kenney	Reg. No. 31,269	Talivaldis Cepuritis	Reg. No. 20,818
Seymour Rothstein	Reg. No. 19,369	Daniel J. Deneufbourg	Reg. No. 33,675
Steven D. Weseman	Reg. No. 41,372	Mark Chao	Reg. No. 37,293
Joseph M. Kuo	Reg. No. 38,943		

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Telephone: (312) 580-1180

PART A: Inventor Information and Signature

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Post Office Address (If different) _____

Inventor's signature: _____ Date: _____

Full name of SECOND joint inventor, if any **Robert Paul**
Citizenship U.S. Residence 519 Stratford Court #S
Del Mar, California 92104
Post Office Address (If different) _____

Second Inventor's signature: _____ Date: _____

PATENT APPLICATION DECLARATION AND POWER OF ATTORNEY

Full name of THIRD joint inventor, if any **Brian Eliceiri**
Citizenship U.S. Residence 3104 Hataca Road
Carlsbad, California 92009
Post Office Address (If different) _____

Second Inventor's signature: _____ Date: _____

PART B: Prior Foreign Application(s)

Serial No.	Country	Day/Month/Year Filed	Priority Claimed
			___ Yes ___ No
			___ Yes ___ No

PART C: Claim for Benefit of Filing Date of Earlier U.S. Application(s)

Serial No.	Filing Date	Status:		
PCT/US99/11780	May 28, 1999	___ Patented	_X_ Pending	___ Abandoned
09/470,881	December 22, 1999	___ Patented	_X_ Pending	___ Abandoned
60/087,220	May 29, 1998	___ Patented	_X_ Pending	___ Abandoned

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                                                         Met Gly
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agc agc aag agc aag ccc aag gac ccc agc cag cgc cgg cgc agc ctg   165
Ser Ser Lys Ser Lys Pro Lys Asp Pro Ser Gln Arg Arg Arg Ser Leu
        5                   10                  15

gag cca ccc gac agc acc cac cac ggg gga ttc cca gcc tcg cag acc   213
Glu Pro Pro Asp Ser Thr His His Gly Gly Phe Pro Ala Ser Gln Thr
    20                  25                  30

ccc aac aag aca gca gcc ccc gac acg cac cgc acc ccc agc cgc tcc   261
Pro Asn Lys Thr Ala Ala Pro Asp Thr His Arg Thr Pro Ser Arg Ser
35                  40                  45                  50

ttt ggg acc gtg gcc acc gag ccc aag ctc ttc ggg ggc ttc aac act   309
Phe Gly Thr Val Ala Thr Glu Pro Lys Leu Phe Gly Gly Phe Asn Thr
                55                  60                  65

tct gac acc gtt acg tcg ccg cag cgt gcc ggg gca ctg gct ggc ggc   357
Ser Asp Thr Val Thr Ser Pro Gln Arg Ala Gly Ala Leu Ala Gly Gly
            70                  75                  80

gtc acc act ttc gtg gct ctc tac gac tac gag tcc cgg act gaa acg   405
Val Thr Thr Phe Val Ala Leu Tyr Asp Tyr Glu Ser Arg Thr Glu Thr
        85                  90                  95

gac ttg tcc ttc aag aaa gga gaa cgc ctg cag att gtc aac aac acg   453
Asp Leu Ser Phe Lys Lys Gly Glu Arg Leu Gln Ile Val Asn Asn Thr
    100                 105                 110

gaa ggt gac tgg tgg ctg gct cat tcc ctc act aca gga cag acg ggc   501
Glu Gly Asp Trp Trp Leu Ala His Ser Leu Thr Thr Gly Gln Thr Gly
115                 120                 125                 130

tac atc ccc agt aac tat gtc gcg ccc tca gac tcc atc cag gct gaa   549
Tyr Ile Pro Ser Asn Tyr Val Ala Pro Ser Asp Ser Ile Gln Ala Glu
                135                 140                 145

gag tgg tac ttt ggg aag atc act cgt cgg gag tcc gag cgg ctg ctg   597
Glu Trp Tyr Phe Gly Lys Ile Thr Arg Arg Glu Ser Glu Arg Leu Leu
            150                 155                 160

ctc aac ccc gaa aac ccc cgg gga acc ttc ttg gtc cgg gag agc gag   645
Leu Asn Pro Glu Asn Pro Arg Gly Thr Phe Leu Val Arg Glu Ser Glu
        165                 170                 175

acg aca aaa ggt gcc tat tgc ctc tcc gtt tct gac ttt gac aac gcc   693
Thr Thr Lys Gly Ala Tyr Cys Leu Ser Val Ser Asp Phe Asp Asn Ala
    180                 185                 190
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aag ggg ctc aat gtg aag cac tac aag atc cgc aag ctg gac agc ggc   741
Lys Gly Leu Asn Val Lys His Tyr Lys Ile Arg Lys Leu Asp Ser Gly
195                 200                 205                 210

ggc ttc tac atc acc tca cgc aca cag ttc agc agc ctg cag cag ctg   789
Gly Phe Tyr Ile Thr Ser Arg Thr Gln Phe Ser Ser Leu Gln Gln Leu
                215                 220                 225

gtg gcc tac tac tcc aaa cat gct gat ggc ttg tgc cac cgc ctg acc   837
Val Ala Tyr Tyr Ser Lys His Ala Asp Gly Leu Cys His Arg Leu Thr
            230                 235                 240

aac gtc tgc ccc acg tcc aag ccc cag acc cag gga ctc gcc aag gac   885
Asn Val Cys Pro Thr Ser Lys Pro Gln Thr Gln Gly Leu Ala Lys Asp
        245                 250                 255

gcg tgg gaa atc ccc cgg gag tcg ctg cgg ctg gag gtg aag ctg ggg   933
Ala Trp Glu Ile Pro Arg Glu Ser Leu Arg Leu Glu Val Lys Leu Gly
    260                 265                 270

cag ggc tgc ttt gga gag gtc tgg atg ggg acc tgg aac ggc acc acc   981
Gln Gly Cys Phe Gly Glu Val Trp Met Gly Thr Trp Asn Gly Thr Thr
275                 280                 285                 290

aga gtg gcc ata aag act ctg aag ccc ggc acc atg tcc ccg gag gcc  1029
Arg Val Ala Ile Lys Thr Leu Lys Pro Gly Thr Met Ser Pro Glu Ala
                295                 300                 305

ttc ctg cag gaa gcc caa gtg atg aag aag ctc cgg cat gag aag ctg  1077
Phe Leu Gln Glu Ala Gln Val Met Lys Lys Leu Arg His Glu Lys Leu
            310                 315                 320

gtt cag ctg tac gca gtg gtg tcg gaa gag ccc atc tac atc gtc act  1125
Val Gln Leu Tyr Ala Val Val Ser Glu Glu Pro Ile Tyr Ile Val Thr
        325                 330                 335

gag tac atg agc aag ggg agc ctc ctg gat ttc ctg aag gga gag atg  1173
Glu Tyr Met Ser Lys Gly Ser Leu Leu Asp Phe Leu Lys Gly Glu Met
    340                 345                 350

ggc aag tac ctg cgg ctg cca cag ctc gtc gat atg gct gct cag att  1221
Gly Lys Tyr Leu Arg Leu Pro Gln Leu Val Asp Met Ala Ala Gln Ile
355                 360                 365                 370

gca tcc ggc atg gcc tat gtg gag agg atg aac tac gtg cac cga gac  1269
Ala Ser Gly Met Ala Tyr Val Glu Arg Met Asn Tyr Val His Arg Asp
                375                 380                 385

ctg cgg gcg gcc aac atc ctg gtg ggg gag aac ctg gtg tgc aag gtg  1317
Leu Arg Ala Ala Asn Ile Leu Val Gly Glu Asn Leu Val Cys Lys Val
            390                 395                 400
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gct gac ttt ggg ctg gca cgc ctc atc gag gac aac gag tac aca gca   1365
Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp Asn Glu Tyr Thr Ala
        405                 410                 415

cgg caa ggt gcc aag ttc ccc atc aag tgg aca gcc ccc gag gca gcc   1413
Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu Ala Ala
    420                 425                 430

ctc tat ggc cgg ttc acc atc aag tcg gat gtc tgg tcc ttc ggc atc   1461
Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser Phe Gly Ile
435                 440                 445                 450

ctg ctg act gag ctg acc acc aag ggc cgg gtg cca tac cca ggg atg   1509
Leu Leu Thr Glu Leu Thr Thr Lys Gly Arg Val Pro Tyr Pro Gly Met
                455                 460                 465

gtc aac agg gag gtg ctg gac cag gtg gag agg ggc tac cgc atg ccc   1557
Val Asn Arg Glu Val Leu Asp Gln Val Glu Arg Gly Tyr Arg Met Pro
            470                 475                 480

tgc ccg ccc gag tgc ccc gag tcg ctg cat gac ctc atg tgc cag tgc   1605
Cys Pro Pro Glu Cys Pro Glu Ser Leu His Asp Leu Met Cys Gln Cys
        485                 490                 495

tgg cgg agg gac cct gag gag cgg ccc act ttt gag tac ctg cag gcc   1653
Trp Arg Arg Asp Pro Glu Glu Arg Pro Thr Phe Glu Tyr Leu Gln Ala
    500                 505                 510

ttc ctg gag gac tac ttc acc tcg aca gag ccc cag tac cag cct gga   1701
Phe Leu Glu Asp Tyr Phe Thr Ser Thr Glu Pro Gln Tyr Gln Pro Gly
515                 520                 525                 530

gag aac cta taggcctgga gctcctcctg gaccagaggc ctcgctgtgg ggtacaggg 1759
Glu Asn Leu
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Ser Leu Glu Pro Pro Asp Ser Thr His His Gly Gly Phe Pro Ala Ser
            20                  25                  30

Gln Thr Pro Asn Lys Thr Ala Ala Pro Asp Thr His Arg Thr Pro Ser
        35                  40                  45

Arg Ser Phe Gly Thr Val Ala Thr Glu Pro Lys Leu Phe Gly Gly Phe
    50                  55                  60
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Asn Thr Ser Asp Thr Val Thr Ser Pro Gln Arg Ala Gly Ala Leu Ala
 65                  70                  75                  80

Gly Gly Val Thr Thr Phe Val Ala Leu Tyr Asp Tyr Glu Ser Arg Thr
                 85                  90                  95

Glu Thr Asp Leu Ser Phe Lys Lys Gly Glu Arg Leu Gln Ile Val Asn
            100                 105                 110

Asn Thr Glu Gly Asp Trp Trp Leu Ala His Ser Leu Thr Thr Gly Gln
        115                 120                 125

Thr Gly Tyr Ile Pro Ser Asn Tyr Val Ala Pro Ser Asp Ser Ile Gln
    130                 135                 140

Ala Glu Glu Trp Tyr Phe Gly Lys Ile Thr Arg Arg Glu Ser Glu Arg
145                 150                 155                 160

Leu Leu Leu Asn Pro Glu Asn Pro Arg Gly Thr Phe Leu Val Arg Glu
                165                 170                 175

Ser Glu Thr Thr Lys Gly Ala Tyr Cys Leu Ser Val Ser Asp Phe Asp
            180                 185                 190

Asn Ala Lys Gly Leu Asn Val Lys His Tyr Lys Ile Arg Lys Leu Asp
        195                 200                 205

Ser Gly Gly Phe Tyr Ile Thr Ser Arg Thr Gln Phe Ser Ser Leu Gln
    210                 215                 220

Gln Leu Val Ala Tyr Tyr Ser Lys His Ala Asp Gly Leu Cys His Arg
225                 230                 235                 240

Leu Thr Asn Val Cys Pro Thr Ser Lys Pro Gln Thr Gln Gly Leu Ala
                245                 250                 255

Lys Asp Ala Trp Glu Ile Pro Arg Glu Ser Leu Arg Leu Glu Val Lys
            260                 265                 270

Leu Gly Gln Gly Cys Phe Gly Glu Val Trp Met Gly Thr Trp Asn Gly
        275                 280                 285

Thr Thr Arg Val Ala Ile Lys Thr Leu Lys Pro Gly Thr Met Ser Pro
    290                 295                 300

Glu Ala Phe Leu Gln Glu Ala Gln Val Met Lys Lys Leu Arg His Glu
305                 310                 315                 320

Lys Leu Val Gln Leu Tyr Ala Val Val Ser Glu Glu Pro Ile Tyr Ile
                325                 330                 335

Val Thr Glu Tyr Met Ser Lys Gly Ser Leu Leu Asp Phe Leu Lys Gly
            340                 345                 350
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Glu Met Gly Lys Tyr Leu Arg Leu Pro Gln Leu Val Asp Met Ala Ala
355 360 365

Gln Ile Ala Ser Gly Met Ala Tyr Val Glu Arg Met Asn Tyr Val His
370 375 380

Arg Asp Leu Arg Ala Ala Asn Ile Leu Val Gly Glu Asn Leu Val Cys
385 390 395 400

Lys Val Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp Asn Glu Tyr
405 410 415

Thr Ala Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu
420 425 430

Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser Phe
435 440 445

Gly Ile Leu Leu Thr Glu Leu Thr Thr Lys Gly Arg Val Pro Tyr Pro
450 455 460

Gly Met Val Asn Arg Glu Val Leu Asp Gln Val Glu Arg Gly Tyr Arg
465 470 475 480

Met Pro Cys Pro Pro Glu Cys Pro Glu Ser Leu His Asp Leu Met Cys
485 490 495

Gln Cys Trp Arg Arg Asp Pro Glu Glu Arg Pro Thr Phe Glu Tyr Leu
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Pro Gly Glu Asn Leu
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agctcctgag aag atg tca gca ata cag gcc gcc tgg cca tcc ggt aca    169
               Met Ser Ala Ile Gln Ala Ala Trp Pro Ser Gly Thr
                 1               5                  10

gaa tgt att gcc aag tac aac ttc cac ggc act gcc gag cag gac ctg   217
Glu Cys Ile Ala Lys Tyr Asn Phe His Gly Thr Ala Glu Gln Asp Leu
         15                  20                  25

ccc ttc tgc aaa gga gac gtg ctc acc att gtg gcc gtc acc aag gac   265
Pro Phe Cys Lys Gly Asp Val Leu Thr Ile Val Ala Val Thr Lys Asp
     30                  35                  40

ccc aac tgg tac aaa gcc aaa aac aag gtg ggc cgt gag ggc atc atc   313
Pro Asn Trp Tyr Lys Ala Lys Asn Lys Val Gly Arg Glu Gly Ile Ile
 45                  50                  55                  60

cca gcc aac tac gtc cag aag cgg gag ggc gtg aag gcg ggt acc aaa   361
Pro Ala Asn Tyr Val Gln Lys Arg Glu Gly Val Lys Ala Gly Thr Lys
                 65                  70                  75

ctc agc ctc atg cct tgg ttc cac ggc aag atc aca cgg gag cag gct   409
Leu Ser Leu Met Pro Trp Phe His Gly Lys Ile Thr Arg Glu Gln Ala
             80                  85                  90

gag cgg ctt ctg tac ccg ccg gag aca ggc ctg ttc ctg gtg cgg gag   457
Glu Arg Leu Leu Tyr Pro Pro Glu Thr Gly Leu Phe Leu Val Arg Glu
         95                  100                 105

agc acc aac tac ccc gga gac tac acg ctg tgc gtg agc tgc gac ggc   505
Ser Thr Asn Tyr Pro Gly Asp Tyr Thr Leu Cys Val Ser Cys Asp Gly
     110                 115                 120

aag gtg gag cac tac cgc atc atg tac cat gcc agc aag ctc agc atc   553
Lys Val Glu His Tyr Arg Ile Met Tyr His Ala Ser Lys Leu Ser Ile
125                 130                 135                 140

gac gag gag gtg tac ttt gag aac ctc atg cag ctg gtg gag cac tac   601
Asp Glu Glu Val Tyr Phe Glu Asn Leu Met Gln Leu Val Glu His Tyr
                145                 150                 155

acc tca gac gca gat gga ctc tgt acg cgc ctc att aaa cca aag gtc   649
Thr Ser Asp Ala Asp Gly Leu Cys Thr Arg Leu Ile Lys Pro Lys Val
            160                 165                 170

atg gag ggc aca gtg gcg gcc cag gat gag ttc tac cgc agc ggc tgg   697
Met Glu Gly Thr Val Ala Ala Gln Asp Glu Phe Tyr Arg Ser Gly Trp
        175                 180                 185
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gcc ctg aac atg aag gag ctg aag ctg ctg cag acc atc ggg aag ggg   745
Ala Leu Asn Met Lys Glu Leu Lys Leu Leu Gln Thr Ile Gly Lys Gly
    190                 195                 200

gag ttc gga gac gtg atg ctg ggc gat tac cga ggg aac aaa gtc gcc   793
Glu Phe Gly Asp Val Met Leu Gly Asp Tyr Arg Gly Asn Lys Val Ala
205                 210                 215                 220

gtc aag tgc att aag aac gac gcc act gcc cag gcc ttc ctg gct gaa   841
Val Lys Cys Ile Lys Asn Asp Ala Thr Ala Gln Ala Phe Leu Ala Glu
                225                 230                 235

gcc tca gtc atg acg caa ctg cgg cat agc aac ctg gtg cag ctc ctg   889
Ala Ser Val Met Thr Gln Leu Arg His Ser Asn Leu Val Gln Leu Leu
            240                 245                 250

ggc gtg atc gtg gag gag aag ggc ggg ctc tac atc gtc act gag tac   937
Gly Val Ile Val Glu Glu Lys Gly Gly Leu Tyr Ile Val Thr Glu Tyr
        255                 260                 265

atg gcc aag ggg agc ctt gtg gac tac ctg cgg tct agg ggt cgg tca   985
Met Ala Lys Gly Ser Leu Val Asp Tyr Leu Arg Ser Arg Gly Arg Ser
    270                 275                 280

gtg ctg ggc gga gac tgt ctc ctc aag ttc tcg cta gat gtc tgc gag   1033
Val Leu Gly Gly Asp Cys Leu Leu Lys Phe Ser Leu Asp Val Cys Glu
285                 290                 295                 300

gcc atg gaa tac ctg gag ggc aac aat ttc gtg cat cga gac ctg gct   1081
Ala Met Glu Tyr Leu Glu Gly Asn Asn Phe Val His Arg Asp Leu Ala
                305                 310                 315

gcc cgc aat gtg ctg gtg tct gag gac aac gtg gcc aag gtc agc gac   1129
Ala Arg Asn Val Leu Val Ser Glu Asp Asn Val Ala Lys Val Ser Asp
            320                 325                 330

ttt ggt ctc acc aag gag gcg tcc agc acc cag gac acg ggc aag ctg   1177
Phe Gly Leu Thr Lys Glu Ala Ser Ser Thr Gln Asp Thr Gly Lys Leu
        335                 340                 345

cca gtc aag tgg aca gcc cct gag gcc ctg aga gag aag aaa ttc tcc   1225
Pro Val Lys Trp Thr Ala Pro Glu Ala Leu Arg Glu Lys Lys Phe Ser
    350                 355                 360

act aag tct gac gtg tgg agt ttc gga atc ctt ctc tgg gaa atc tac   1273
Thr Lys Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile Tyr
365                 370                 375                 380

tcc ttt ggg cga gtg cct tat cca aga att ccc ctg aag gac gtc gtc   1321
Ser Phe Gly Arg Val Pro Tyr Pro Arg Ile Pro Leu Lys Asp Val Val
                385                 390                 395
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cct cgg gtg gag aag ggc tac aag atg gat gcc ccc gac ggc tgc ccg 1369
Pro Arg Val Glu Lys Gly Tyr Lys Met Asp Ala Pro Asp Gly Cys Pro
400 405 410

ccc gca gtc tat gaa gtc atg aag aac tgc tgg cac ctg gac gcc gcc 1417
Pro Ala Val Tyr Glu Val Met Lys Asn Cys Trp His Leu Asp Ala Ala
415 420 425

atg cgg ccc tcc ttc cta cag ctc cga gag cag ctt gag cac atc aaa 1465
Met Arg Pro Ser Phe Leu Gln Leu Arg Glu Gln Leu Glu His Ile Lys
430 435 440

acc cac gag ctg cac ctg tgacggctgg cctccgcctg ggtcatgggc1513
Thr His Glu Leu His Leu
445 450

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Lys Ala Lys Asn Lys Val Gly Arg Glu Gly Ile Ile Pro Ala Asn Tyr
     50                  55                  60

Val Gln Lys Arg Glu Gly Val Lys Ala Gly Thr Lys Leu Ser Leu Met
 65                  70                  75                  80

Pro Trp Phe His Gly Lys Ile Thr Arg Glu Gln Ala Glu Arg Leu Leu
                 85                  90                  95

Tyr Pro Pro Glu Thr Gly Leu Phe Leu Val Arg Glu Ser Thr Asn Tyr
            100                 105                 110

Pro Gly Asp Tyr Thr Leu Cys Val Ser Cys Asp Gly Lys Val Glu His
        115                 120                 125

Tyr Arg Ile Met Tyr His Ala Ser Lys Leu Ser Ile Asp Glu Glu Val
    130                 135                 140

Tyr Phe Glu Asn Leu Met Gln Leu Val Glu His Tyr Thr Ser Asp Ala
145                 150                 155                 160

Asp Gly Leu Cys Thr Arg Leu Ile Lys Pro Lys Val Met Glu Gly Thr
                165                 170                 175

Val Ala Ala Gln Asp Glu Phe Tyr Arg Ser Gly Trp Ala Leu Asn Met
            180                 185                 190

Lys Glu Leu Lys Leu Leu Gln Thr Ile Gly Lys Gly Glu Phe Gly Asp
        195                 200                 205

Val Met Leu Gly Asp Tyr Arg Gly Asn Lys Val Ala Val Lys Cys Ile
    210                 215                 220

Lys Asn Asp Ala Thr Ala Gln Ala Phe Leu Ala Glu Ala Ser Val Met
225                 230                 235                 240

Thr Gln Leu Arg His Ser Asn Leu Val Gln Leu Leu Gly Val Ile Val
                245                 250                 255

Glu Glu Lys Gly Gly Leu Tyr Ile Val Thr Glu Tyr Met Ala Lys Gly
            260                 265                 270

Ser Leu Val Asp Tyr Leu Arg Ser Arg Gly Arg Ser Val Leu Gly Gly
        275                 280                 285

Asp Cys Leu Leu Lys Phe Ser Leu Asp Val Cys Glu Ala Met Glu Tyr
    290                 295                 300

Leu Glu Gly Asn Asn Phe Val His Arg Asp Leu Ala Ala Arg Asn Val
305                 310                 315                 320
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Leu Val Ser Glu Asp Asn Val Ala Lys Val Ser Asp Phe Gly Leu Thr
325 330 335

Lys Glu Ala Ser Ser Thr Gln Asp Thr Gly Lys Leu Pro Val Lys Trp
340 345 350

Thr Ala Pro Glu Ala Leu Arg Glu Lys Lys Phe Ser Thr Lys Ser Asp
355 360 365

Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile Tyr Ser Phe Gly Arg
370 375 380

Val Pro Tyr Pro Arg Ile Pro Leu Lys Asp Val Val Pro Arg Val Glu
385 390 395 400

Lys Gly Tyr Lys Met Asp Ala Pro Asp Gly Cys Pro Pro Ala Val Tyr
405 410 415

Glu Val Met Lys Asn Cys Trp His Leu Asp Ala Ala Met Arg Pro Ser
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Phe Leu Gln Leu Arg Glu Gln Leu Glu His Ile Lys Thr His Glu Leu
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His Leu
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gtaggcgccg gaaggctggg ggccccggag ccgggccggc gtggcccgag ttccggtgag 180

cggacggcgg cgcgcgcaga tttgata atg ggc tgc att aaa agt aaa gaa aac 234
Met Gly Cys Ile Lys Ser Lys Glu Asn
1 5

aaa agt cca gcc att aaa tac aga cct gaa aat act cca gag cct gtc 282
Lys Ser Pro Ala Ile Lys Tyr Arg Pro Glu Asn Thr Pro Glu Pro Val
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agt aca agt gtg agc cat tat gga gca gaa ccc act aca gtg tca cca 330
Ser Thr Ser Val Ser His Tyr Gly Ala Glu Pro Thr Thr Val Ser Pro
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Cys Pro Ser Ser Ser Ala Lys Gly Thr Ala Val Asn Phe Ser Ser Leu
45 50 55

tcc atg aca cca ttt gga gga tcc tca ggg gta acg cct ttt gga ggt 426
Ser Met Thr Pro Phe Gly Gly Ser Ser Gly Val Thr Pro Phe Gly Gly
60 65 70

gca tct tcc tca ttt tca gtg gtg cca agt tca tat cct gct ggt tta 474
Ala Ser Ser Ser Phe Ser Val Val Pro Ser Ser Tyr Pro Ala Gly Leu
75 80 85

aca ggt ggt gtt act ata ttt gtg gcc tta tat gat tat gaa gct aga 522
Thr Gly Gly Val Thr Ile Phe Val Ala Leu Tyr Asp Tyr Glu Ala Arg
90 95 100 105

act aca gaa gac ctt tca ttt aag aag ggt gaa aga ttt caa ata att 570
Thr Thr Glu Asp Leu Ser Phe Lys Lys Gly Glu Arg Phe Gln Ile Ile
110 115 120

aac aat acg gaa gga gat tgg tgg gaa gca aga tca atc gct aca gga 618
Asn Asn Thr Glu Gly Asp Trp Trp Glu Ala Arg Ser Ile Ala Thr Gly
125 130 135

aag aat ggt tat atc ccg agc aat tat gta gcg cct gca gat tcc att 666
Lys Asn Gly Tyr Ile Pro Ser Asn Tyr Val Ala Pro Ala Asp Ser Ile
140 145 150

cag gca gaa gaa tgg tat ttt ggc aaa atg ggg aga aaa gat gct gaa 714
Gln Ala Glu Glu Trp Tyr Phe Gly Lys Met Gly Arg Lys Asp Ala Glu
155 160 165

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aga tta ctt ttg aat cct gga aat caa cga ggt att ttc tta gta aga   762
Arg Leu Leu Leu Asn Pro Gly Asn Gln Arg Gly Ile Phe Leu Val Arg
170                 175                 180                 185

gag agt gaa aca act aaa ggt gct tat tcc ctt tct att cgt gat tgg   810
Glu Ser Glu Thr Thr Lys Gly Ala Tyr Ser Leu Ser Ile Arg Asp Trp
                190                 195                 200

gat gag ata agg ggt gac aat gtg aaa cac tac aaa att agg aaa ctt   858
Asp Glu Ile Arg Gly Asp Asn Val Lys His Tyr Lys Ile Arg Lys Leu
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gac aat ggt gga tac tat atc aca acc aga gca caa ttt gat act ctg   906
Asp Asn Gly Gly Tyr Tyr Ile Thr Thr Arg Ala Gln Phe Asp Thr Leu
        220                 225                 230

cag aaa ttg gtg aaa cac tac aca gaa cat gct gat ggt tta tgc cac   954
Gln Lys Leu Val Lys His Tyr Thr Glu His Ala Asp Gly Leu Cys His
    235                 240                 245

aag ttg aca act gtg tgt cca act gtg aaa cct cag act caa ggt cta   1002
Lys Leu Thr Thr Val Cys Pro Thr Val Lys Pro Gln Thr Gln Gly Leu
250                 255                 260                 265

gca aaa gat gct tgg gaa atc cct cga gaa tct ttg cga cta gag gtt   1050
Ala Lys Asp Ala Trp Glu Ile Pro Arg Glu Ser Leu Arg Leu Glu Val
                270                 275                 280

aaa cta gga caa gga tgt ttc ggc gaa gtg tgg atg gga aca tgg aat   1098
Lys Leu Gly Gln Gly Cys Phe Gly Glu Val Trp Met Gly Thr Trp Asn
            285                 290                 295

gga acc acg aaa gta gca atc aaa aca cta aaa cca ggt aca atg atg   1146
Gly Thr Thr Lys Val Ala Ile Lys Thr Leu Lys Pro Gly Thr Met Met
        300                 305                 310

cca gaa gct ttc ctt caa gaa gct cag ata atg aaa aaa tta aga cat   1194
Pro Glu Ala Phe Leu Gln Glu Ala Gln Ile Met Lys Lys Leu Arg His
    315                 320                 325

gat aaa ctt gtt cca cta tat gct gtt gtt tct gaa gaa cca att tac   1242
Asp Lys Leu Val Pro Leu Tyr Ala Val Val Ser Glu Glu Pro Ile Tyr
330                 335                 340                 345

att gtc act gaa ttt atg tca aaa gga agc tta tta gat ttc ctt aag   1290
Ile Val Thr Glu Phe Met Ser Lys Gly Ser Leu Leu Asp Phe Leu Lys
                350                 355                 360

gaa gga gat gga aag tat ttg aag ctt cca cag ctg gtt gat atg gct   1338
Glu Gly Asp Gly Lys Tyr Leu Lys Leu Pro Gln Leu Val Asp Met Ala
            365                 370                 375
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gct cag att gct gat ggt atg gca tat att gaa aga atg aac tat att   1386
Ala Gln Ile Ala Asp Gly Met Ala Tyr Ile Glu Arg Met Asn Tyr Ile
        380                 385                 390

cac cga gat ctt cgg gct gct aat att ctt gta gga gaa aat ctt gtg   1434
His Arg Asp Leu Arg Ala Ala Asn Ile Leu Val Gly Glu Asn Leu Val
    395                 400                 405

tgc aaa ata gca gac ttt ggt tta gca agg tta att gaa gac aat gaa   1482
Cys Lys Ile Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp Asn Glu
410                 415                 420                 425

tac aca gca aga caa ggt gca aaa ttt cca atc aaa tgg aca gct cct   1530
Tyr Thr Ala Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro
                430                 435                 440

gaa gct gca ctg tat ggt cgg ttt aca ata aag tct gat gtc tgg tca   1578
Glu Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser
            445                 450                 455

ttt gga att ctg caa aca gaa cta gta aca aag ggc cga gtg cca tat   1626
Phe Gly Ile Leu Gln Thr Glu Leu Val Thr Lys Gly Arg Val Pro Tyr
        460                 465                 470

cca ggt atg gtg aac cgt gaa gta cta gaa caa gtg gag cga gga tac   1674
Pro Gly Met Val Asn Arg Glu Val Leu Glu Gln Val Glu Arg Gly Tyr
    475                 480                 485

agg atg ccg tgc cct cag ggc tgt cca gaa tcc ctc cat gaa ttg atg   1722
Arg Met Pro Cys Pro Gln Gly Cys Pro Glu Ser Leu His Glu Leu Met
490                 495                 500                 505

aat ctg tgt tgg aag aag gac cct gat gaa aga cca aca ttt gaa tat   1770
Asn Leu Cys Trp Lys Lys Asp Pro Asp Glu Arg Pro Thr Phe Glu Tyr
                510                 515                 520

att cag tcc ttc ttg gaa gac tac ttc act gct aca gag cca cag tac   1818
Ile Gln Ser Phe Leu Glu Asp Tyr Phe Thr Ala Thr Glu Pro Gln Tyr
            525                 530                 535

cag cca gga gaa aat tta taattcaagt agcctatttt atatgcacaa1866
Gln Pro Gly Glu Asn Leu
        540

atctgccaaa atataaagaa cttgtgtaga ttttctacag gaatcaaaag aagaaaatct 1926

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ctttttttc cccaagtatt aaactctaat gtaccaatga tgaatttatc agcgtatttc 2046

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Gly Ala Glu Pro Thr Thr Val Ser Pro Cys Pro Ser Ser Ser Ala Lys
35 40 45

Gly Thr Ala Val Asn Phe Ser Ser Leu Ser Met Thr Pro Phe Gly Gly
50 55 60

Ser Ser Gly Val Thr Pro Phe Gly Gly Ala Ser Ser Ser Phe Ser Val
65 70 75 80

Val Pro Ser Ser Tyr Pro Ala Gly Leu Thr Gly Gly Val Thr Ile Phe
85 90 95

Val Ala Leu Tyr Asp Tyr Glu Ala Arg Thr Thr Glu Asp Leu Ser Phe
100 105 110

Lys Lys Gly Glu Arg Phe Gln Ile Ile Asn Asn Thr Glu Gly Asp Trp
115 120 125

Trp Glu Ala Arg Ser Ile Ala Thr Gly Lys Asn Gly Tyr Ile Pro Ser
130 135 140

Asn Tyr Val Ala Pro Ala Asp Ser Ile Gln Ala Glu Glu Trp Tyr Phe
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Gly Lys Met Gly Arg Lys Asp Ala Glu Arg Leu Leu Leu Asn Pro Gly
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Asn Gln Arg Gly Ile Phe Leu Val Arg Glu Ser Glu Thr Thr Lys Gly
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Ala Tyr Ser Leu Ser Ile Arg Asp Trp Asp Glu Ile Arg Gly Asp Asn
195 200 205

Val Lys His Tyr Lys Ile Arg Lys Leu Asp Asn Gly Gly Tyr Tyr Ile
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Thr Thr Arg Ala Gln Phe Asp Thr Leu Gln Lys Leu Val Lys His Tyr
225 230 235 240

Thr Glu His Ala Asp Gly Leu Cys His Lys Leu Thr Thr Val Cys Pro
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Thr Val Lys Pro Gln Thr Gln Gly Leu Ala Lys Asp Ala Trp Glu Ile
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Gly Glu Val Trp Met Gly Thr Trp Asn Gly Thr Thr Lys Val Ala Ile
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Leu Ala Arg Leu Ile Glu Asp Asn Glu Tyr Thr Ala Arg Gln Gly Ala
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Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu Ala Ala Leu Tyr Gly Arg
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Phe Thr Ile Lys Ser Asp Val Trp Ser Phe Gly Ile Leu Gln Thr Glu
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465                 470                 475                 480

Val Leu Glu Gln Val Glu Arg Gly Tyr Arg Met Pro Cys Pro Gln Gly
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Cys Pro Glu Ser Leu His Glu Leu Met Asn Leu Cys Trp Lys Lys Asp
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Tyr Phe Thr Ala Thr Glu Pro Gln Tyr Gln Pro Gly Glu Asn Leu
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